

# Chemical Assay of Drugs and Drug Metabolites

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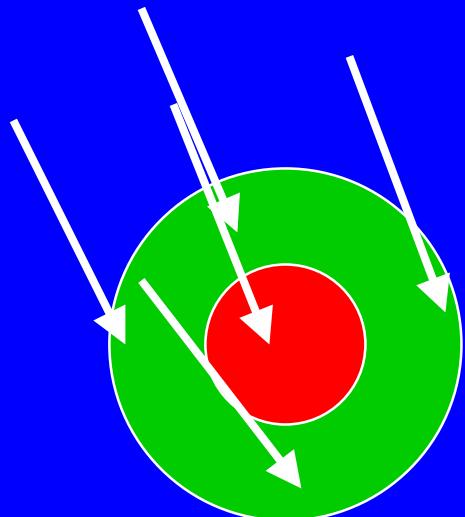
# Lecture Outline

- Quantification principles
  - Analytical PK lab tasks
- Chromatography
- Detection - spectroscopies
  - Optical
  - Mass
- Examples
  - FddA
  - Formoterol
- References

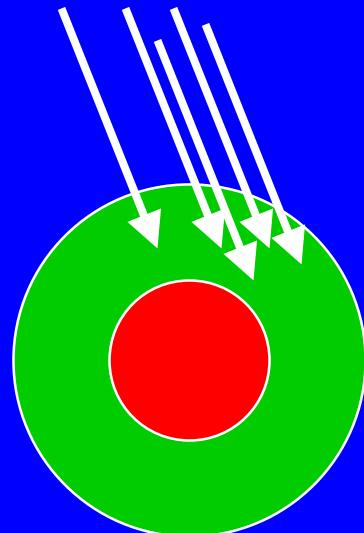
# Definition of Analytical Terms

- Limits of detection (LOD)
  - Sensitivity is the minimum detectable concentration change that can be observed at a specified concentration
  - LOD is the minimum mass or concentration of analyte that can be detected at an acceptable signal to noise (S/N) ratio
- Limits of quantification (LOQ)
  - Analyte mass or concentration required to give an acceptable level of confidence in the measured analyte quantity
  - Always greater (usually 3x) than the minimum LOD

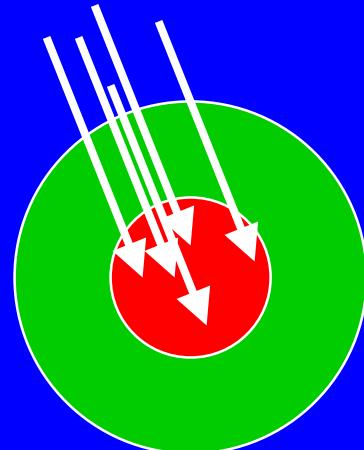
# Accuracy vs. Precision



Good accuracy  
Poor precision



Poor accuracy  
Good precision



Good accuracy  
Good precision

# Pharmaceutical Industry PK Lab

## Analytical Assays (1)

- Parent drug usually the target analyte for Phase 1 dose response and safety determinations
- Scale of runs: 30-50 samples/patient, plus 10-15 standards, procedural blanks, plus 10-15 QC pools or previously analyzed samples
- Several patients per run - effort to optimize patient/(standards + QC) ratio. Result is >100 samples/run
- Analytical runs require automation & rugged instrumentation, continuous operation for assay cycle time X number of samples
- Develop assays on 96 well or 384 well devices

# Pharmaceutical Industry PK Lab

## Analytical Assays (2)

- Speed of assay development principal determinant of methodology choice
- Avoid derivatization chemistry
- Use solid phase extraction or simple methanol/acetonitrile protein precipitation
- Time is money (5 min LC/MS/MS assay vs. 40 min HPLC)
- Cassette dosing (multiple drugs co-administered in single animal) – trendy technique with little merit

# Assay Issues

- What to assay (what is important?)
  - Species -
    - man, non-human primate, rat, mouse (transgenic)
  - Tissue/Fluid
    - liver, target organ, plasma, excreta
  - Isolated organ/tissue fluids
    - liver slices, human liver microsomes, CYPs, other enzymes

# Assay Issues

- Commercial Aides
  - Drug metabolizing preparations
    - Human liver tissue or hepatocytes – all enzymes present in fresh (not frozen) tissue – single use only
    - Microsomes from frozen liver; easily stored
    - Recombinant CYPs and other enzymes - widely available (yeast, baculovirus, bacteria) and some mammalian cells with NADPH CYP reductase
    - CYP substrates, antibodies, inhibitors, inducers
  - Computer software - predict metabolites, pKa, pLogD, logP
  - Contract Research Organizations

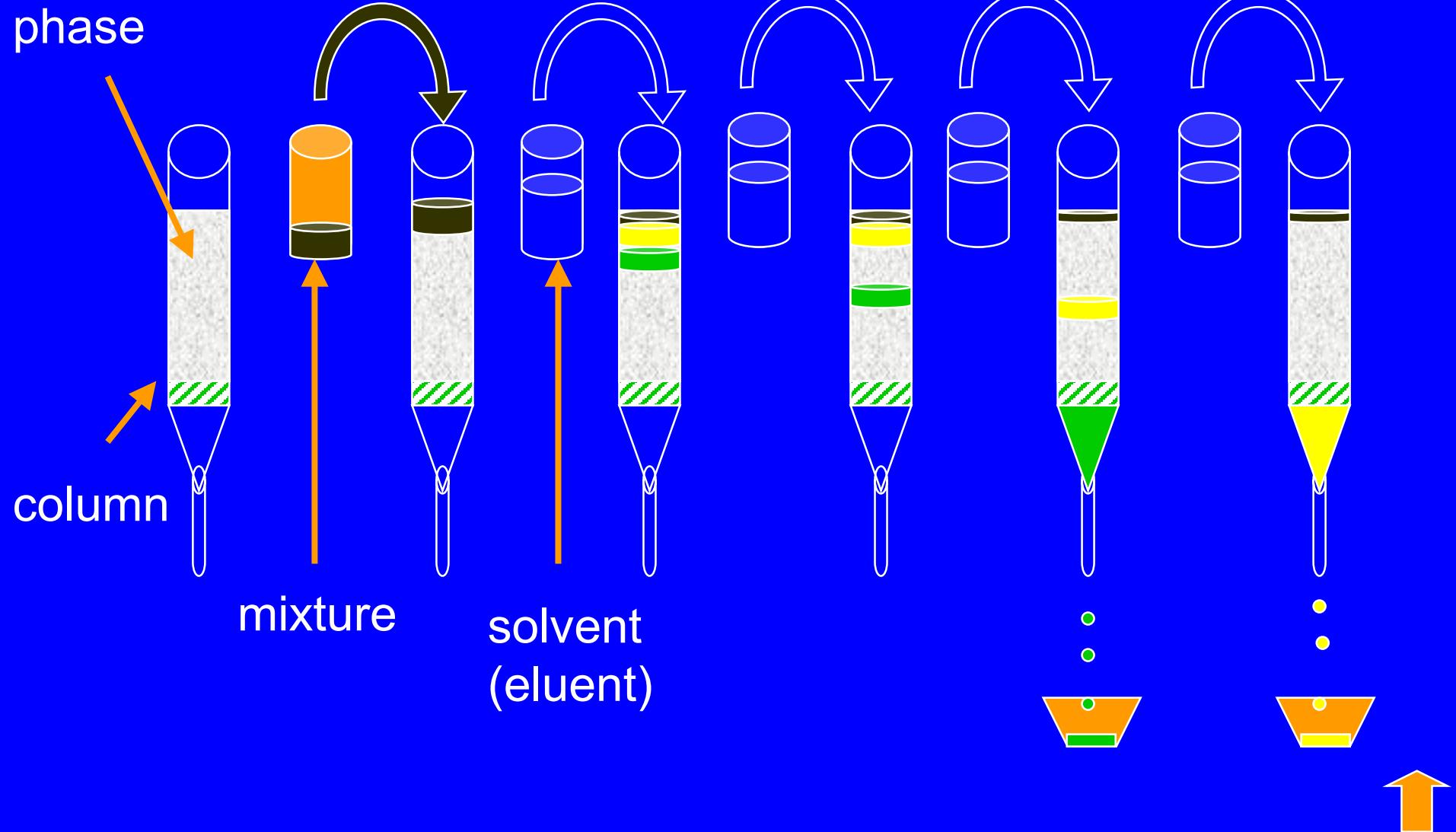
# Separation Methods

- Liquid chromatography
  - High Performance
  - Immunoaffinity
  - Reverse phase
  - Cation, Anion Exchange
  - Size exclusion
- Thin layer chromatography
- Gas chromatography
- Capillary electrophoresis

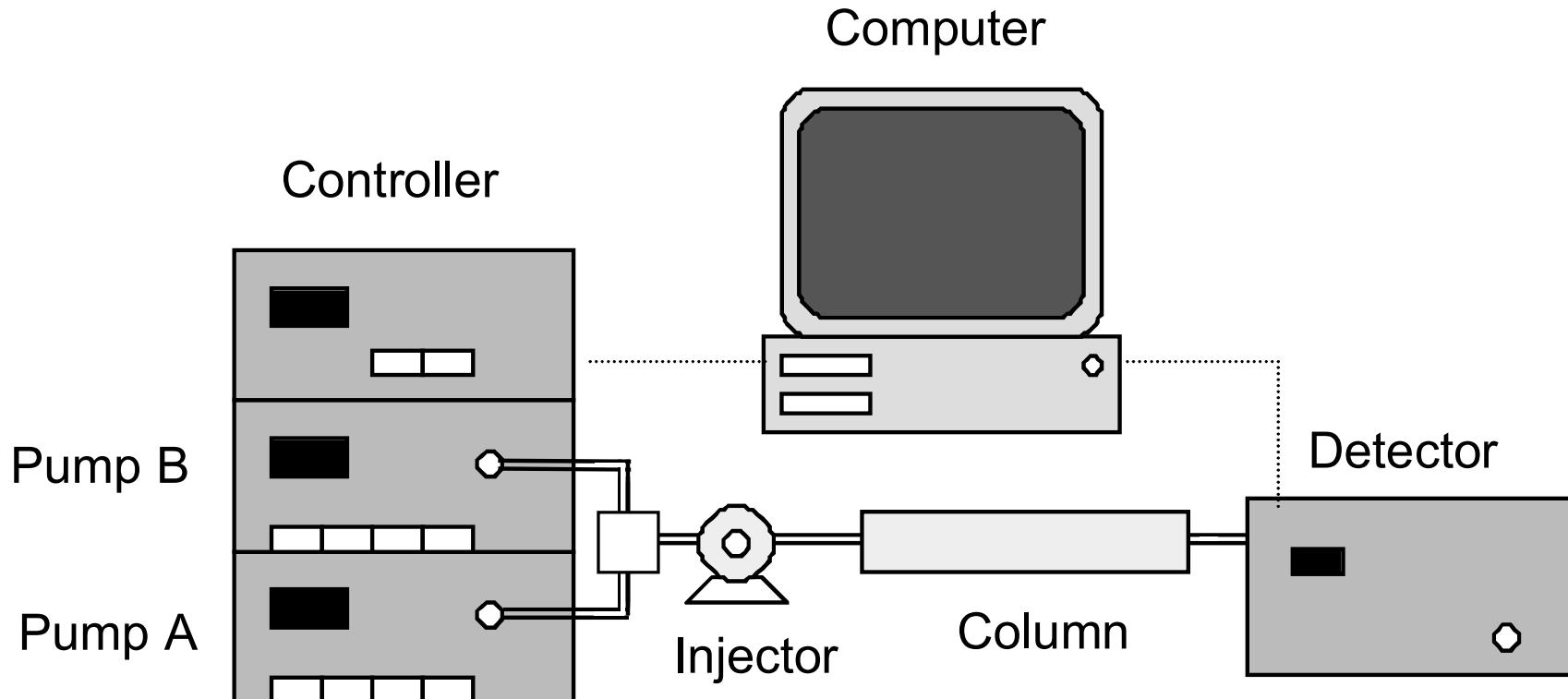


# Liquid Chromatography

stationary  
phase



# HPLC Apparatus

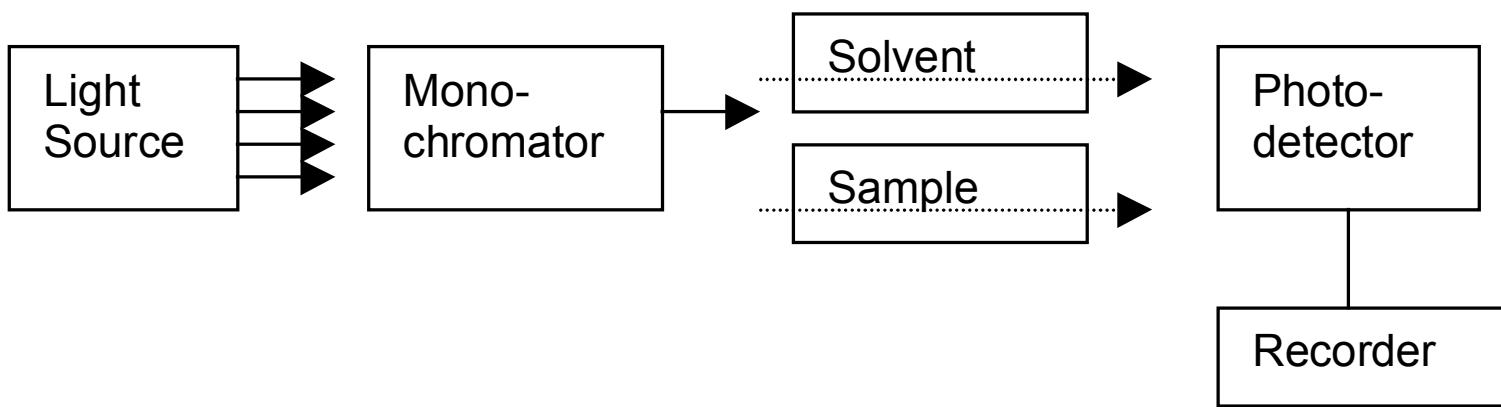


# Detection Principles

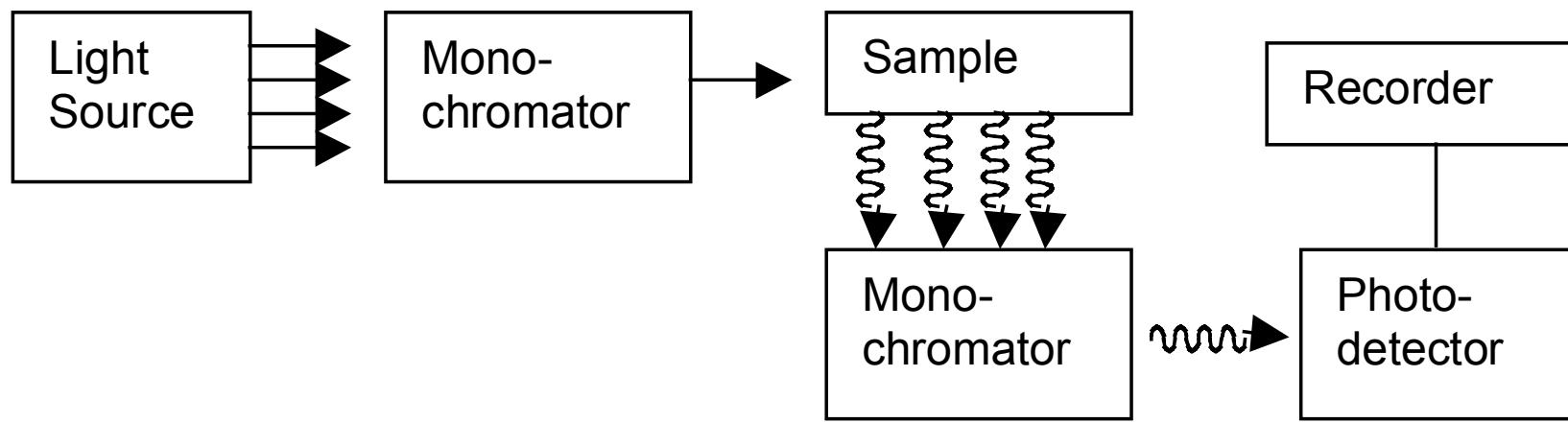
- Ultraviolet or Fluorescence Spectroscopy
  - chromophore in drug or derivatized drug
  - most useful for known target analytes
- Nuclear Magnetic Resonance Spectrometry
  - most useful for totally unknown chemical structure characterization
  - least sensitive
- Mass Spectrometry
  - versatile ionization modes for liquids and gases
    - electron, chemical, electrospray, desorption
  - versatile mass analyzers with varying capabilities
    - magnetic, ion trap, quadrupole, time-of-flight
  - very sensitive and structurally informative – example: air, acetaminophen



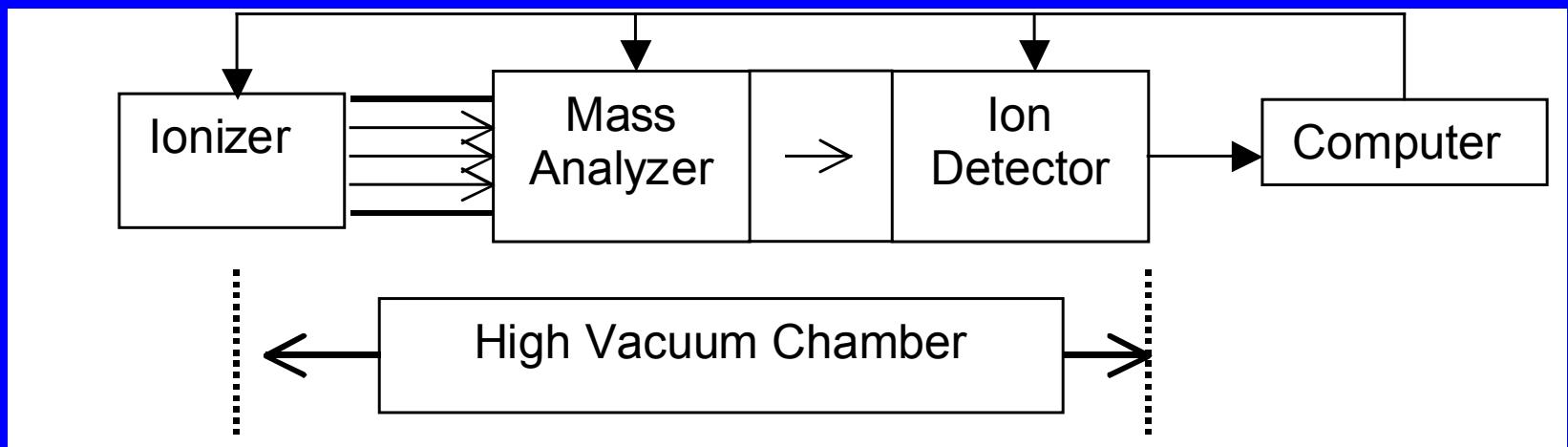
# UV Absorption Spectrophotometer



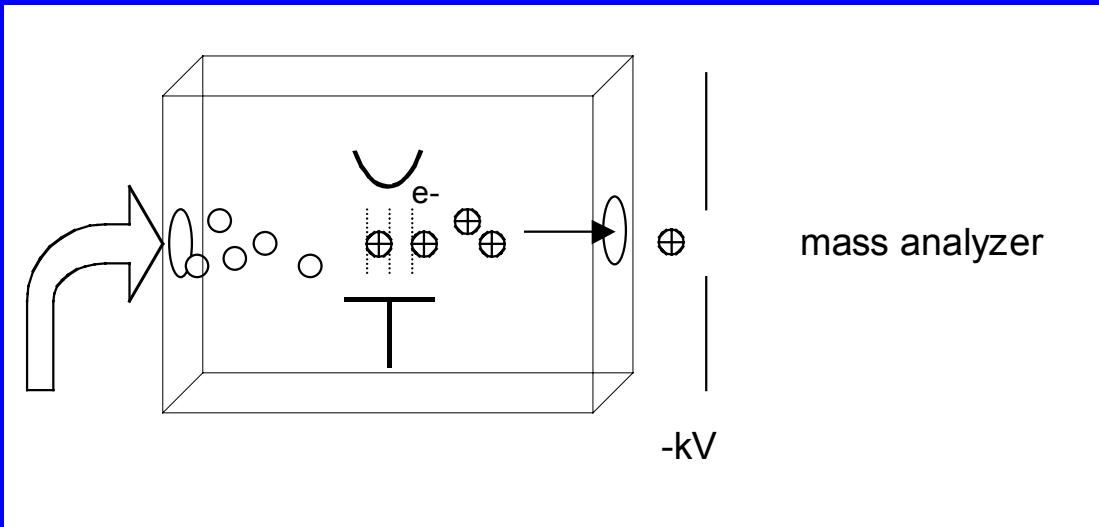
# Emission Spectrophotometer



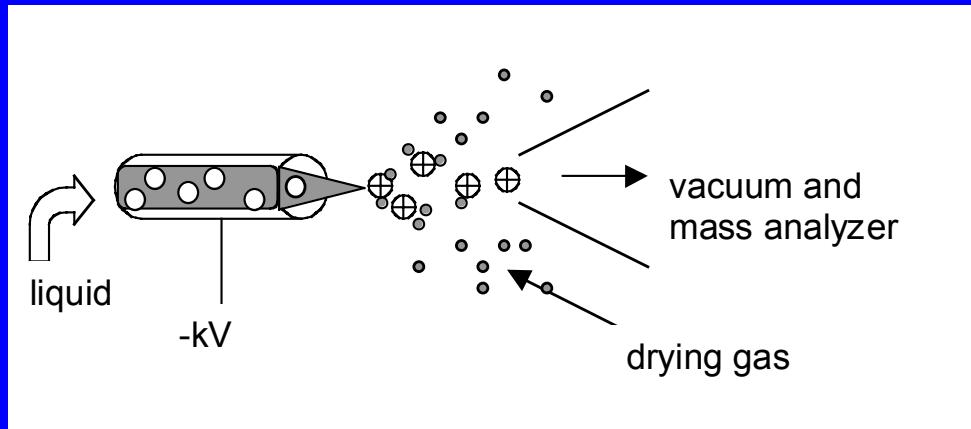
# Mass Spectrometer Component Overview



# Mass Spectrometer Ionizers



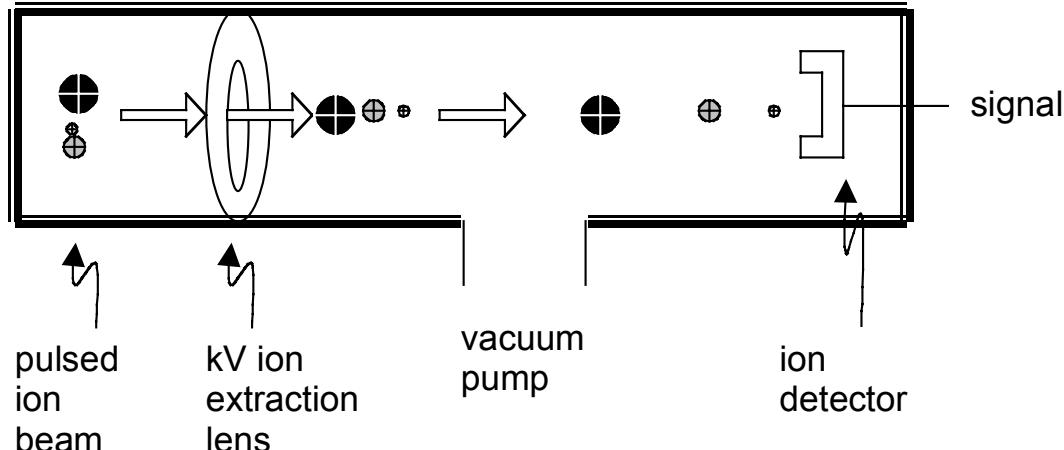
Electron  
Ionization  
(in vacuo)



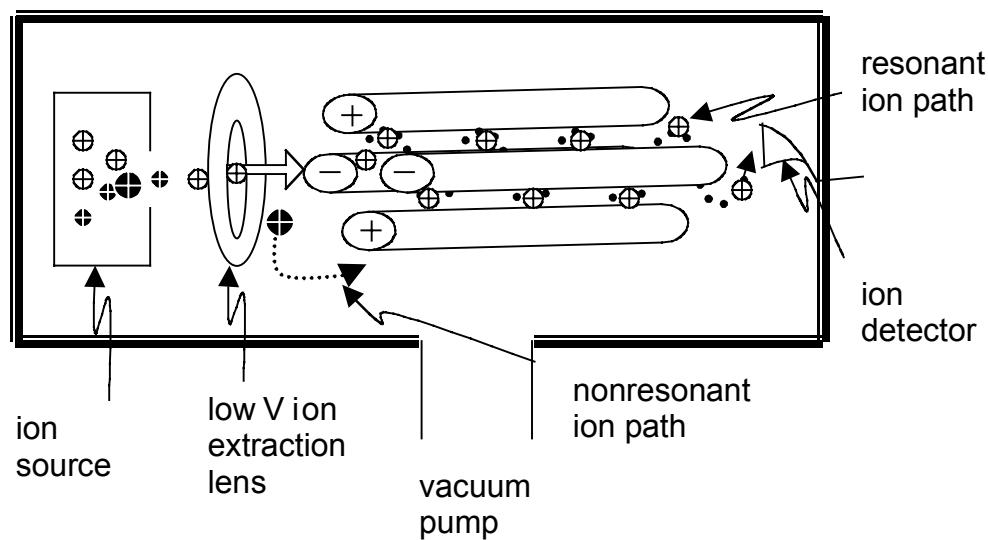
Electrospray  
Ionization  
(external)



# Mass Analyzers



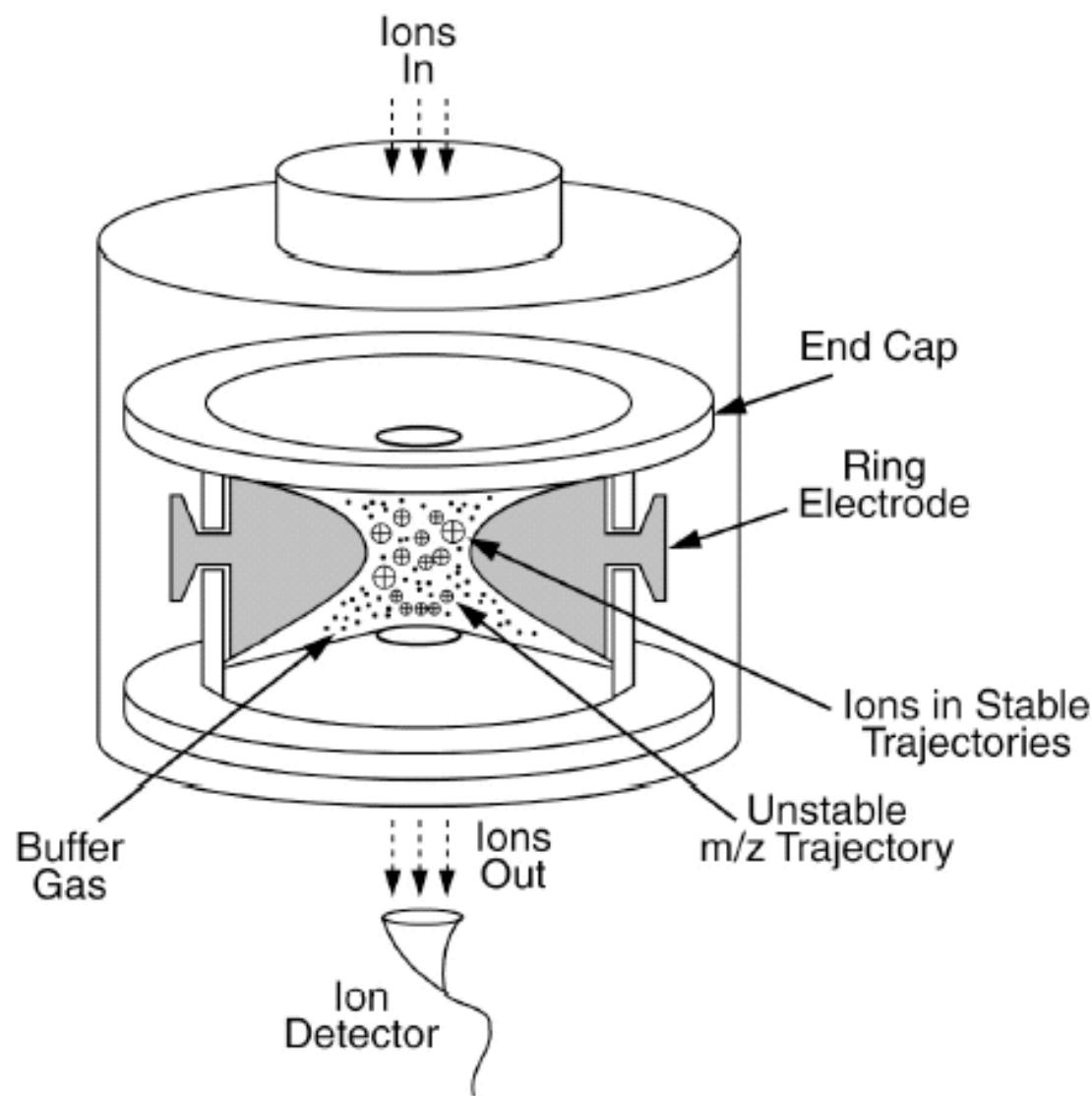
Time-of-flight  
(TOF)



Quadrupole  
(q)

# Quadrupole Ion Trap

C.



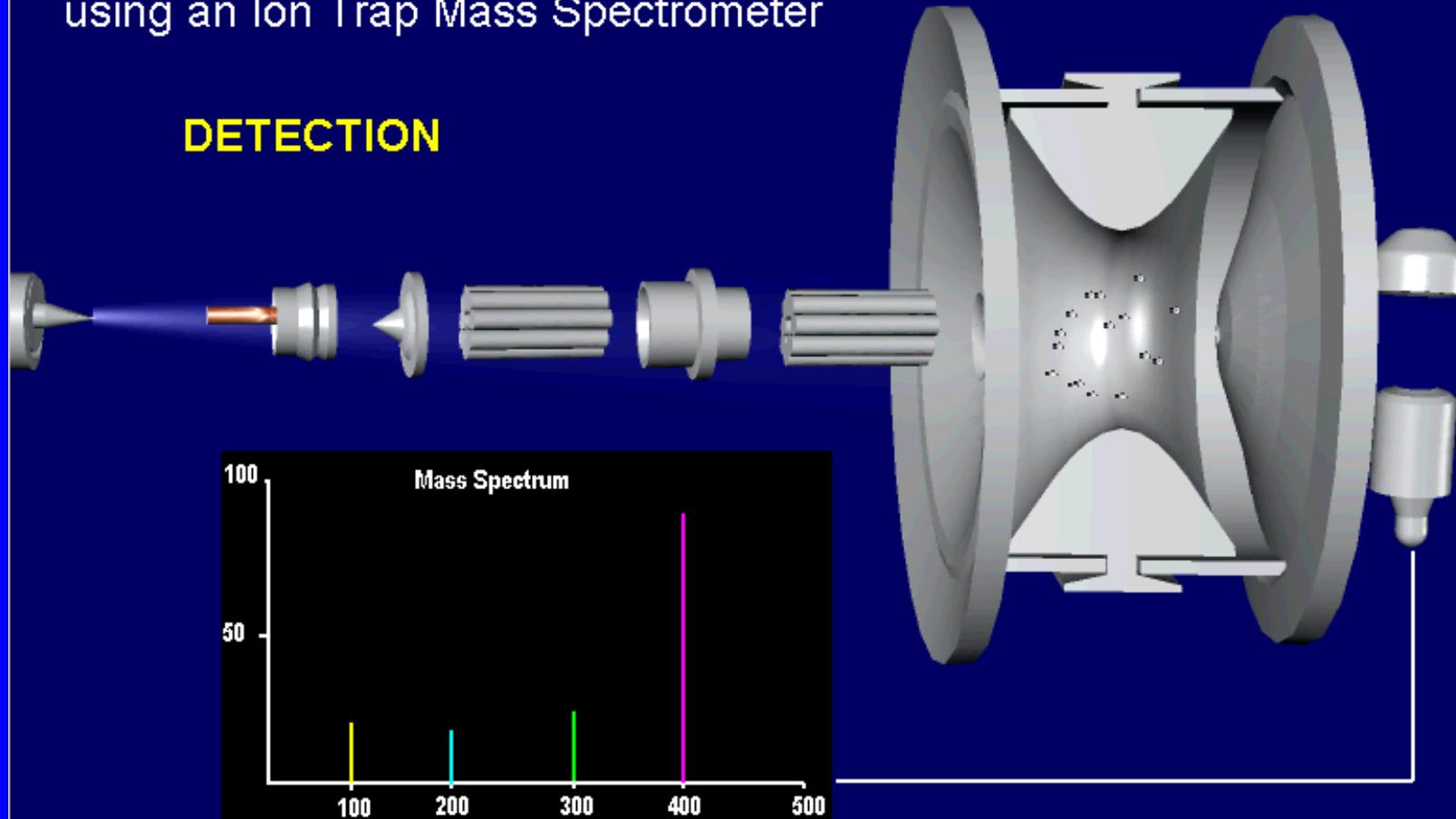
# Electrospray-Ion Trap Mass Spectrometer

esilcq



Overview of Electrospray Ionization  
using an Ion Trap Mass Spectrometer

## DETECTION



TRAPPING

SCANNING

DETECTION



Chap12Fig - Microsoft...

IonTrapDemo

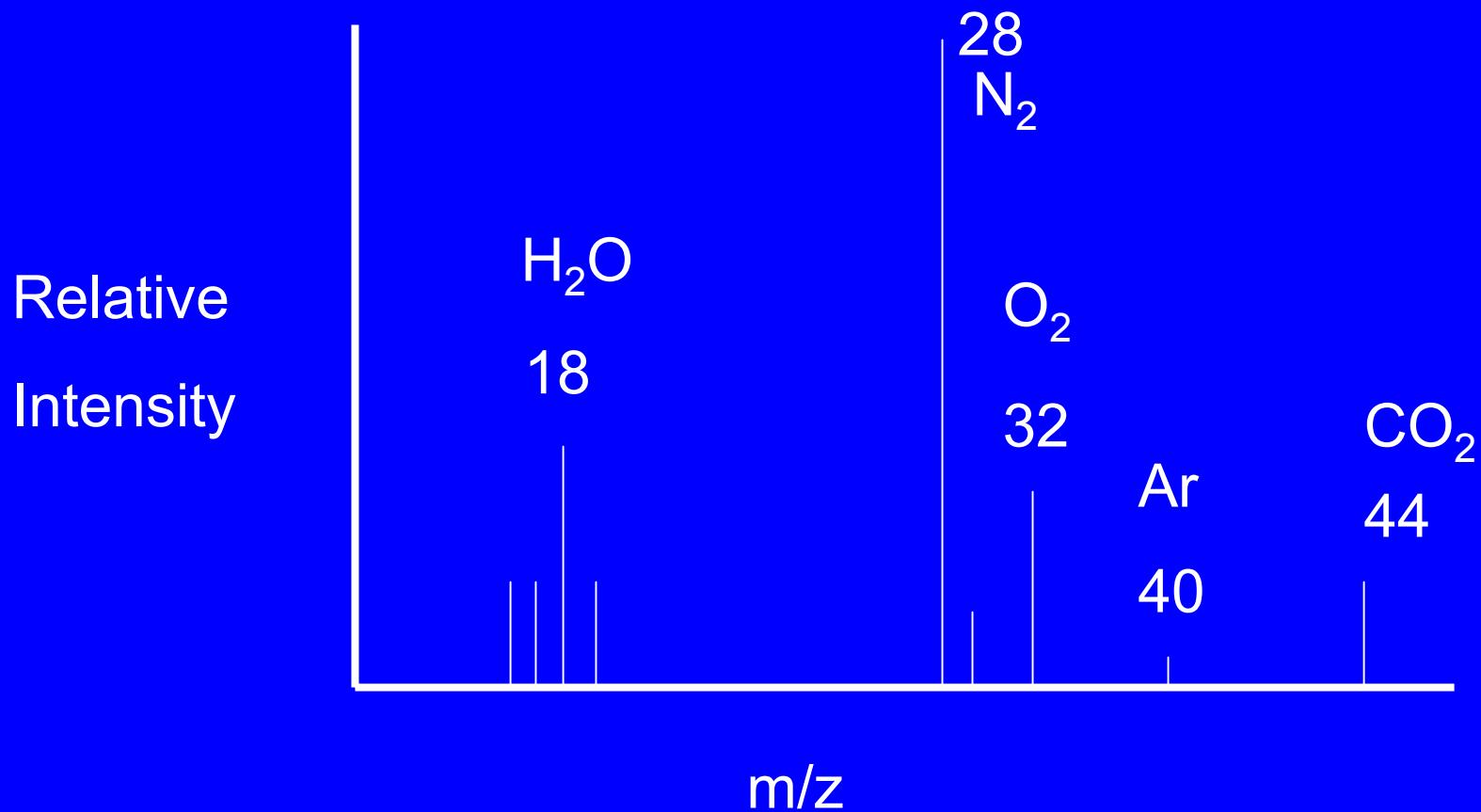
esilcq



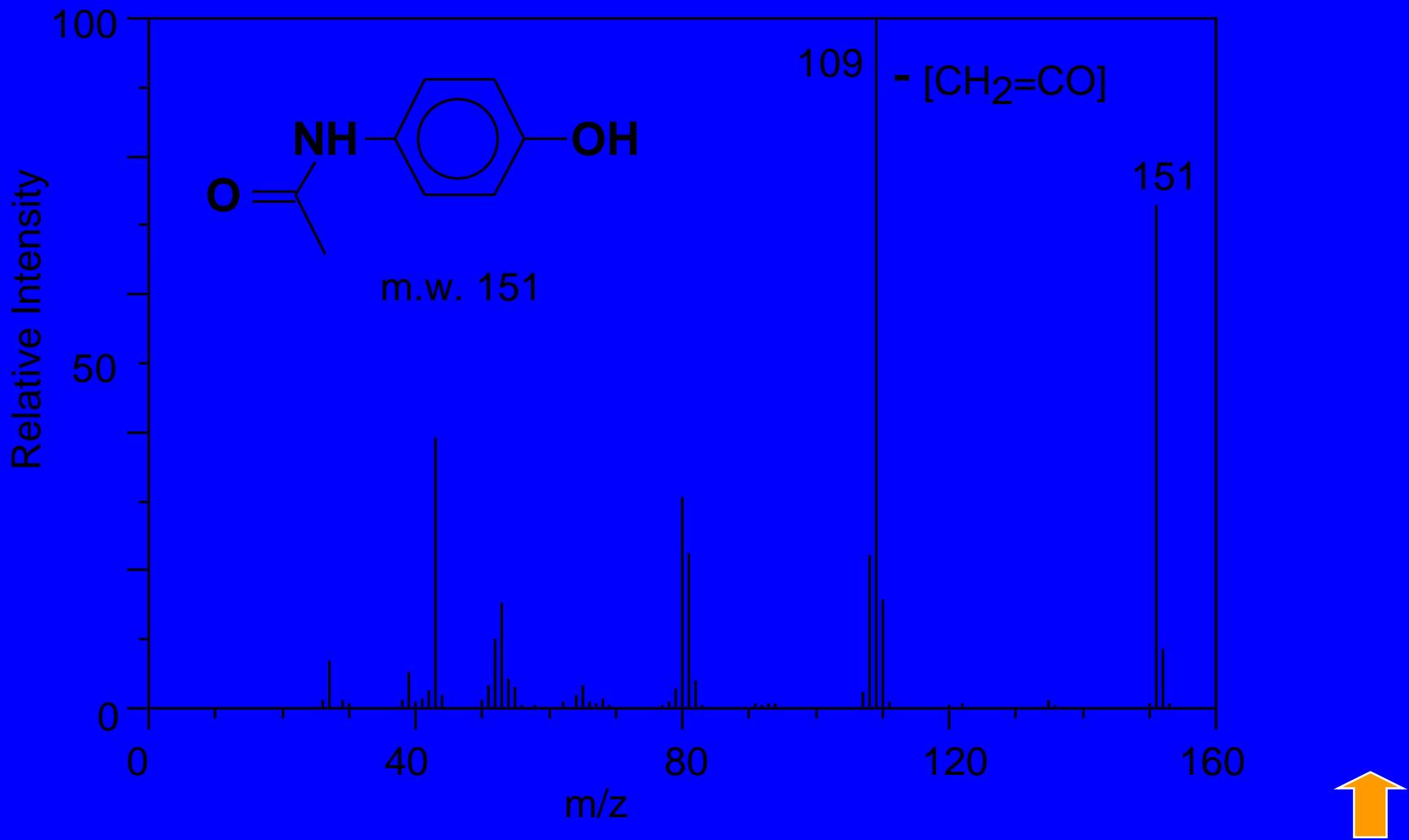
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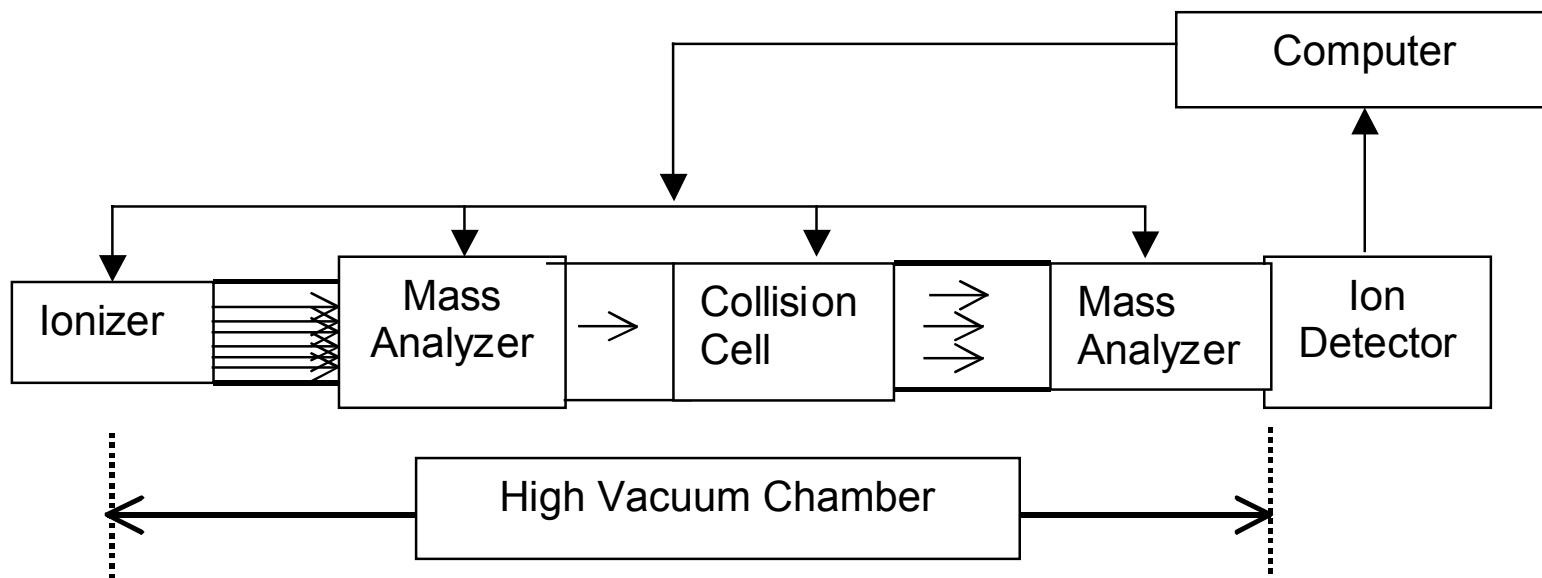
# Mass Spectrum of Air



# Mass spectrum of acetaminophen

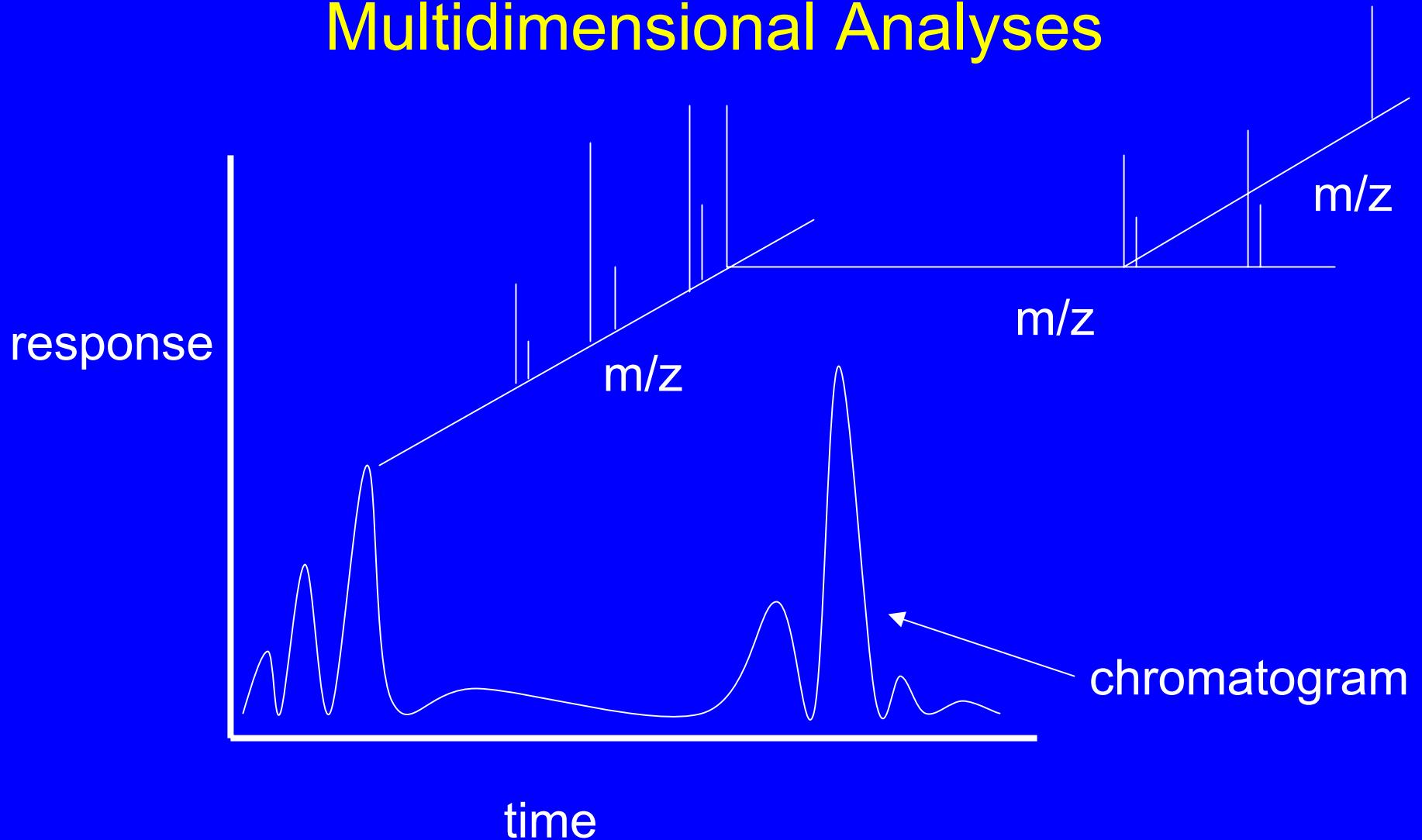


# Tandem Mass Analysis

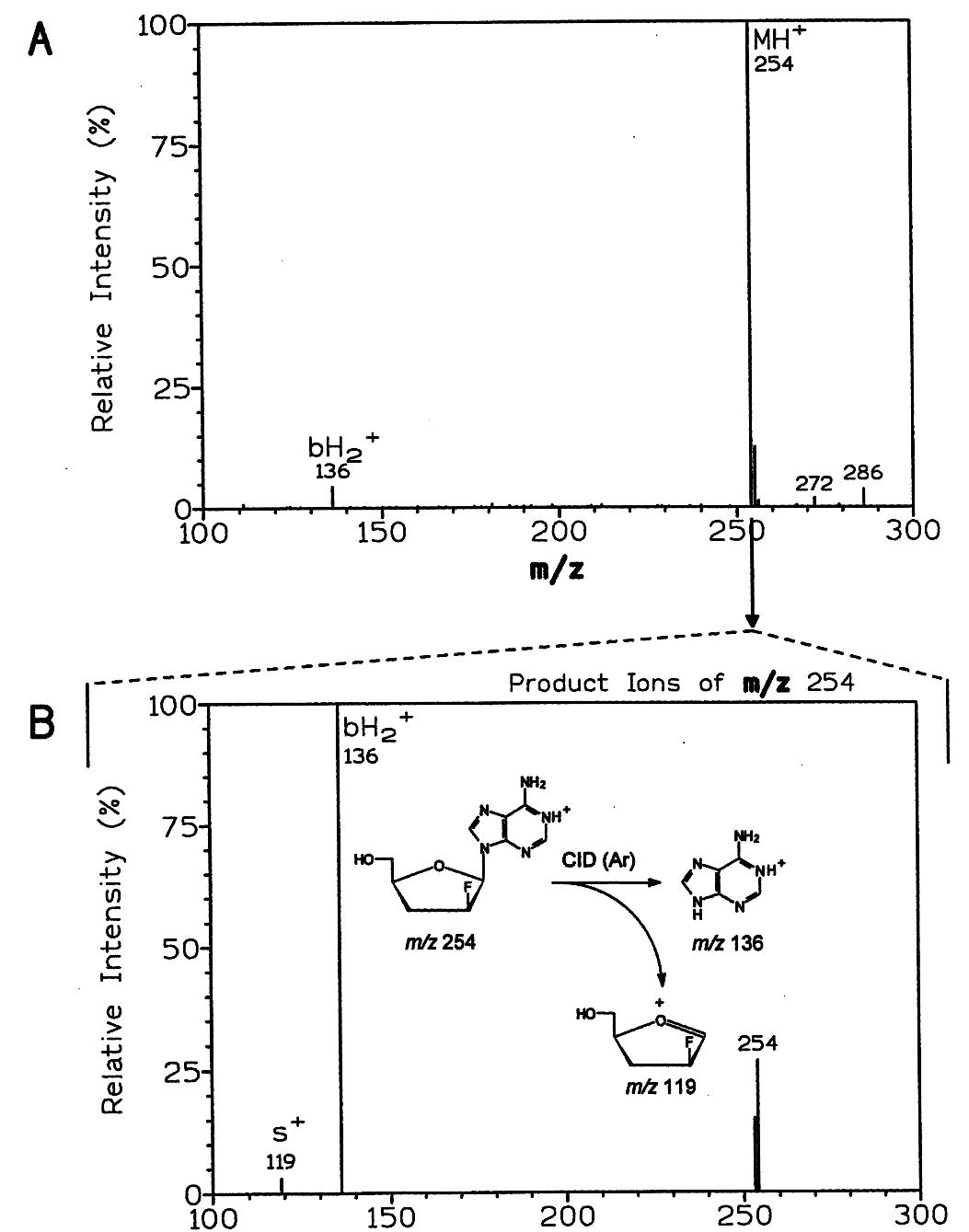


Examples: ESI or APCI with QTOF, QQQ, ITMS

# Multidimensional Analyses



FddA  
MS



MS/MS  
of  
FddA  
 $\text{MH}^+$

# Pharmaceutical Industry PK Lab Analytical Assay Work Load for New Chemical Entities

Method	1990	1998	2000
HPLC	75%	50-60%	20%
GC/MS	12%	3%	2%
LC/MS	3%	40-50%	60-75%
RIA	10%	10%	10%
Preliminary lead profile time	18 m	4 m	0

Conclusion: requirement for speed (not instrumentation cost) dictates choice of analytical methods

# Popular Methods for Qualitative & Quantitative Assays in Clinical Pharmacology

- Enzyme Linked Immunoassay (ELISA)
  - Many 96 well formatted colorimetric or radiometric commercial assay kits for specific compounds
- Fluorescence polarization immunoassay (FPIA)
  - Measures difference in fluorescence between bound and free antigen
  - Important in therapeutic drug monitoring
- HPLC/UV or Fluorescence
  - Very robust & common assay technology – F-dAA
- LC/MS or LC/MS/MS
  - High speed, less requirement for sample preparation
  - F-dAA

# Examples of Analytical Methods Applied in New Drug Research

- 1. FddA
  - HPLC/UV vs HPLC/MS/MS for PK study
- 2. Formoterol
  - Metabolite analyses by HPLC/radiochromatography
  - LC/MS qualitative analyses

# Example 1. Analytical studies for pharmacokinetic measurements in the development of new drugs for AIDS

James A. Kelley (DCT, NCI)

## LC/MS/MS

- Jian Wang
- *Jeri S. Roth*

## PHASE I F-ddA

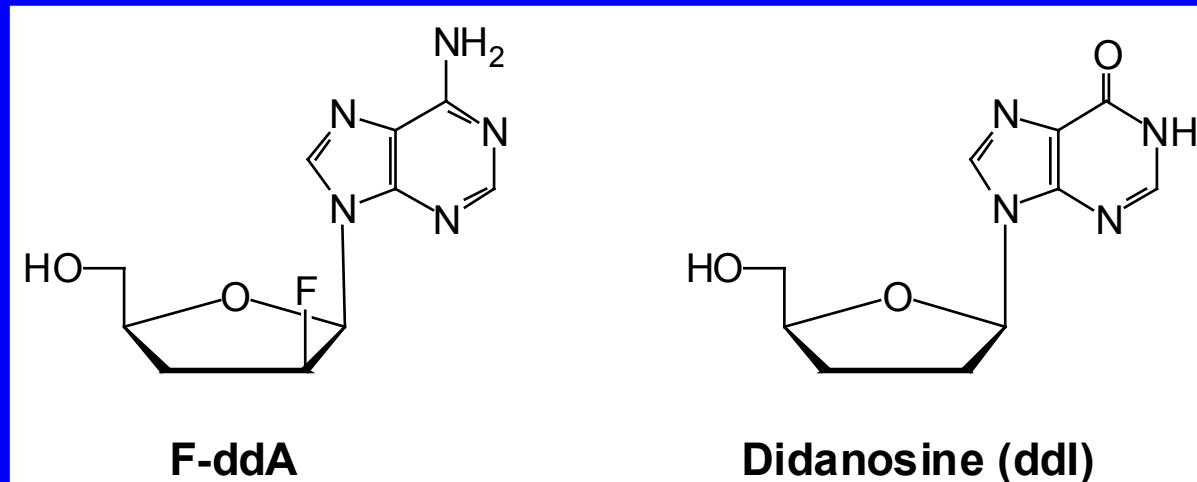
- *Harry Ford, Jr.*
- *Jeri S. Roth*
- *Nancy M. Malinowski*
- Robert Yarchoan  
(HAMB, DCS, NCI)
- Richard F. Little (HAMB, DCS, NCI)

## F-ddATP Analysis

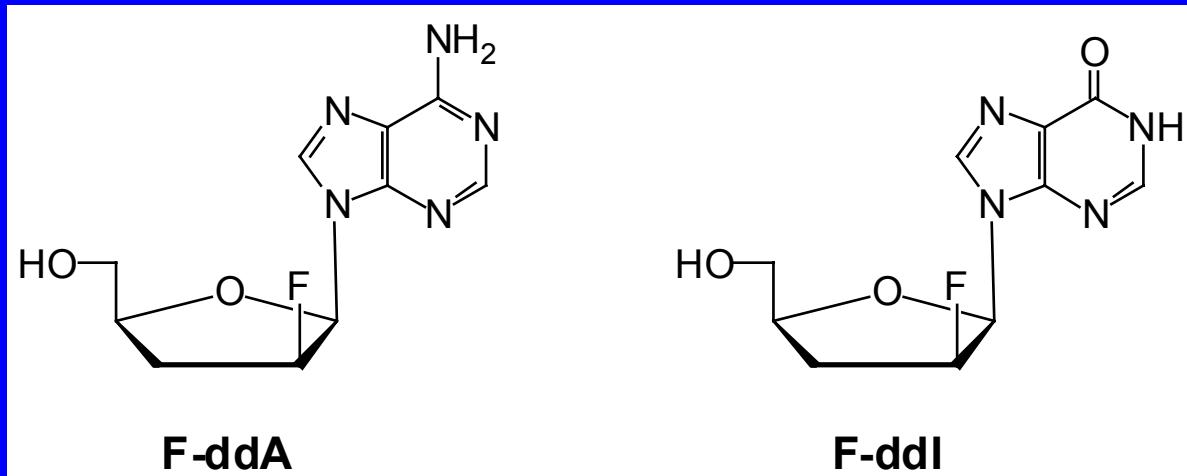
- *Harry Ford, Jr.*
- *Christine Dai*
- Heping Zhang
- Mark F. Kavlick (MB, DCS, NCI)

## FddI Products

- *Jeri S. Roth*
- John S. Driscoll
- Victor E. Marquez
- Robert J. Lutz (BEIP)
- Robert L. Dedrick (BEIP)

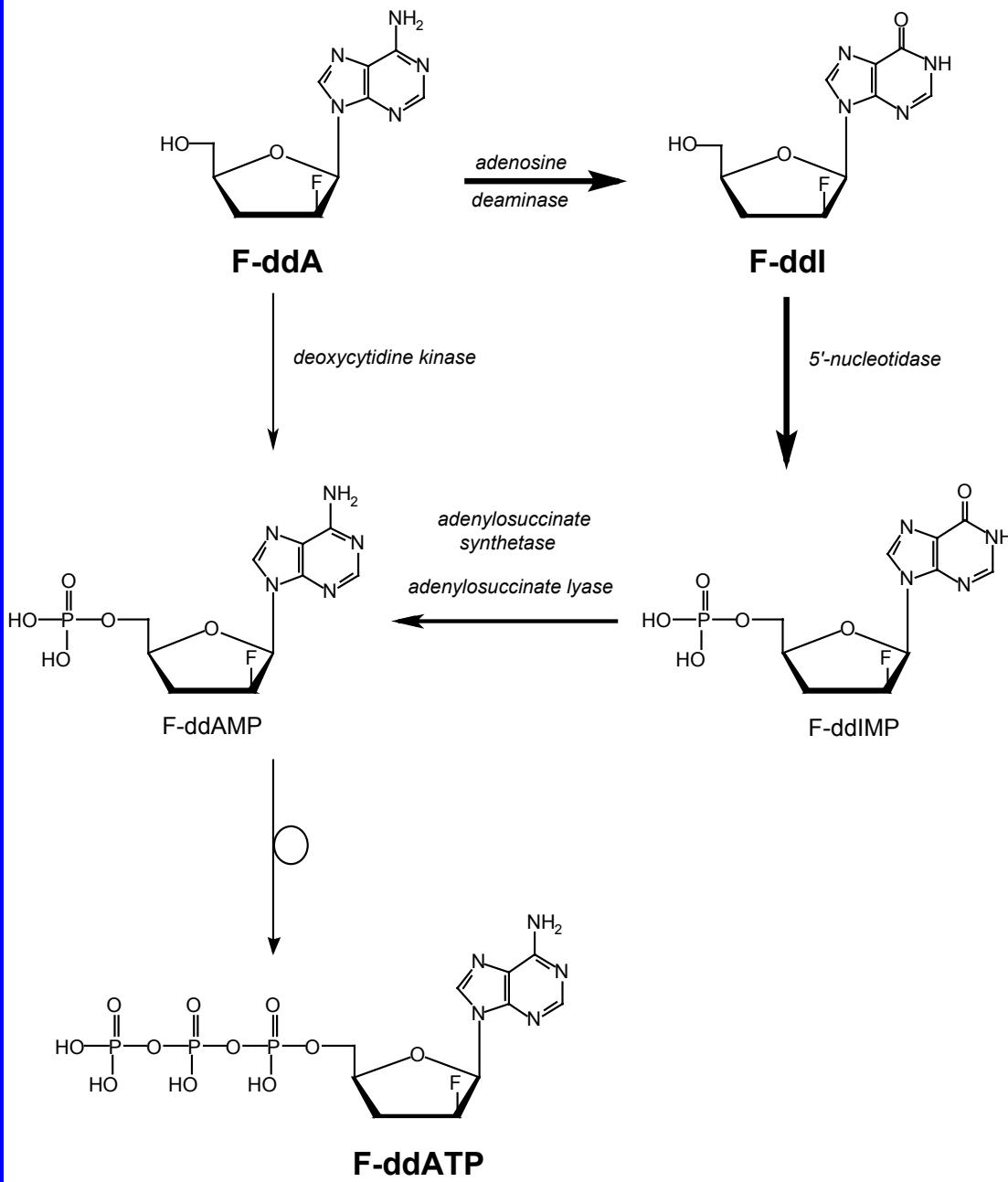


- Synthetic dideoxynucleoside
- Acid stable (pH 1.0, 37 °C)
- More lipophilic than inosine analogue
  - (**Log P = -0.183 versus -1.210**)
- More resistant to metabolic degradation than Didanosine (ddl)
- Inhibitor of HIV reverse transcriptase



- In vitro activity and potency approximate that of ddI (protective over the range 5-100  $\mu$ M)
- Primary metabolite (F-ddI) is also anti-HIV active
- In vitro activity against drug-resistant HIV (AZT, ddI, ddC)
- Good oral bioavailability (mouse, dog, monkey)
- Potential for reduced clinical toxicity
- NCI-sponsored adult Phase I clinical trial started in June 1996 (R. Yarchoan et al., DCS, NCI)

# F-ddA METABOLISM



## F-ddA PK Study

- Is once-a-day administration the optimal dosing regimen for Idenosine (F-ddA)?
- What is the best F-ddI prodrug candidate for optimum drug delivery to the CNS?
- What is the effect of hydroxyurea in combination with Idenosine (F-ddA) on intracellular nucleotide concentrations in patients?
- What concentrations of F-ddATP in patients are associated with reproducible anti-retroviral activity?
- What is the intracellular half-life of F-ddATP and other nucleotide metabolites in vivo?

# F-ddA PHASE I CLINICAL TRIAL

## (Stage 1)



### Treatment Plan:

- Day 1 - 90 min i.v. infusion
- Day 2 - p.o. fasting (liquid)
- Day 3 - p.o. fasting (capsule)
- Day 4 - p.o. food (capsule)
- Day 5 to 84 - p.o. bid (capsule)

### Dose Escalation:

- Level 1 - 0.2 mg/kg (3)
- Level 2 - 0.4 mg/kg (3)
- Level 3 - 0.8 mg/kg (3)
- Level 4 - 1.6 mg/kg (4)
- Level 5 - 3.2 mg/kg (3)**

*(Arrows indicate days of current PK sampling)*

# F-ddA PHASE I CLINICAL TRIAL

## (STAGE 2)



### Treatment Plan:

Day 1 to 28 - F-ddA only  
p.o. daily (capsule)  
(1 hr before breakfast)

Day 29 to 84 - F-ddA p.o. daily (capsule)  
(1 hr before breakfast)  
d4T (40 mg) bid  
Nelfinavir (750 mg) tid  
(with food)

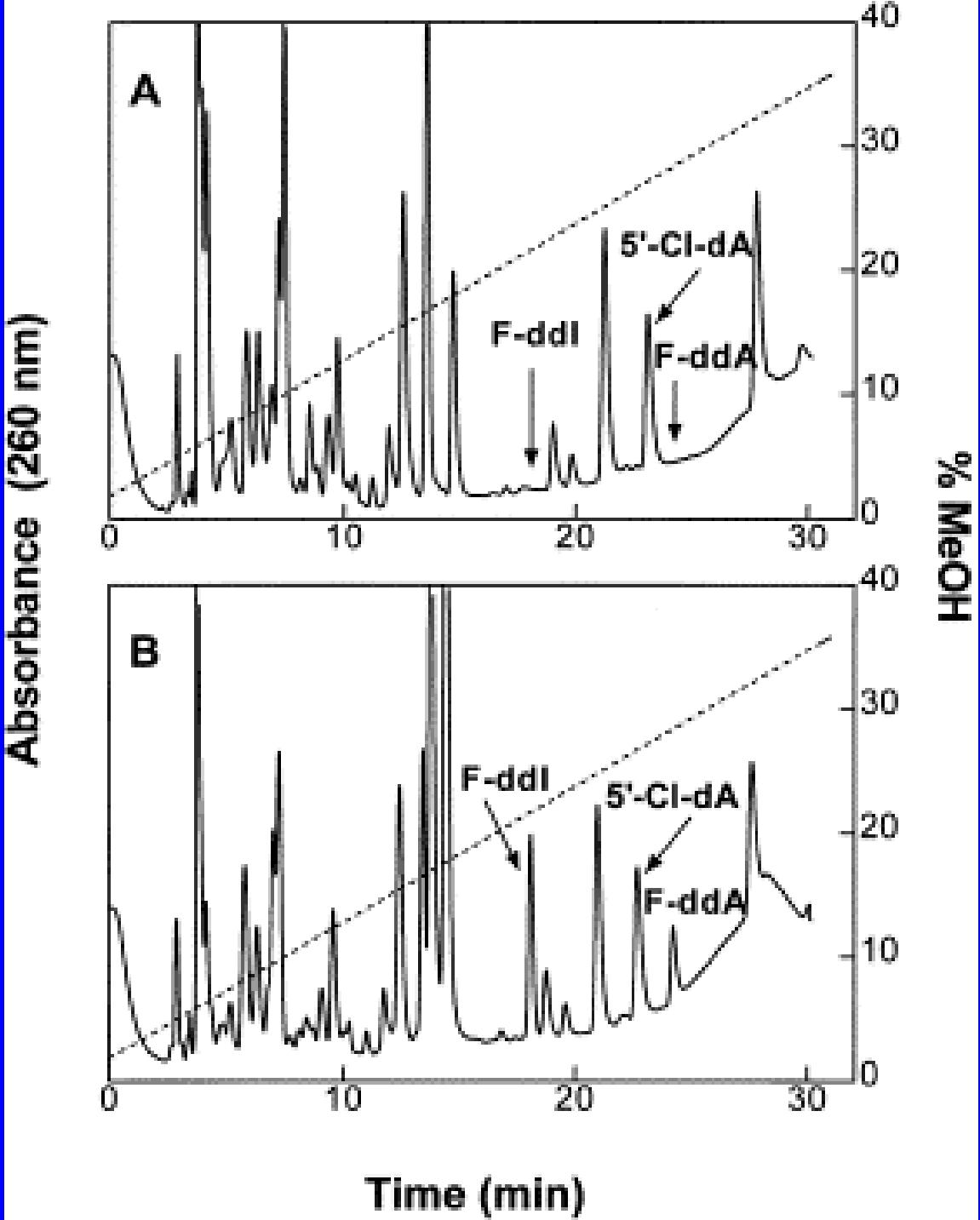
### Dose Escalation:

**Level 6 - 3.2 mg/kg (4)**

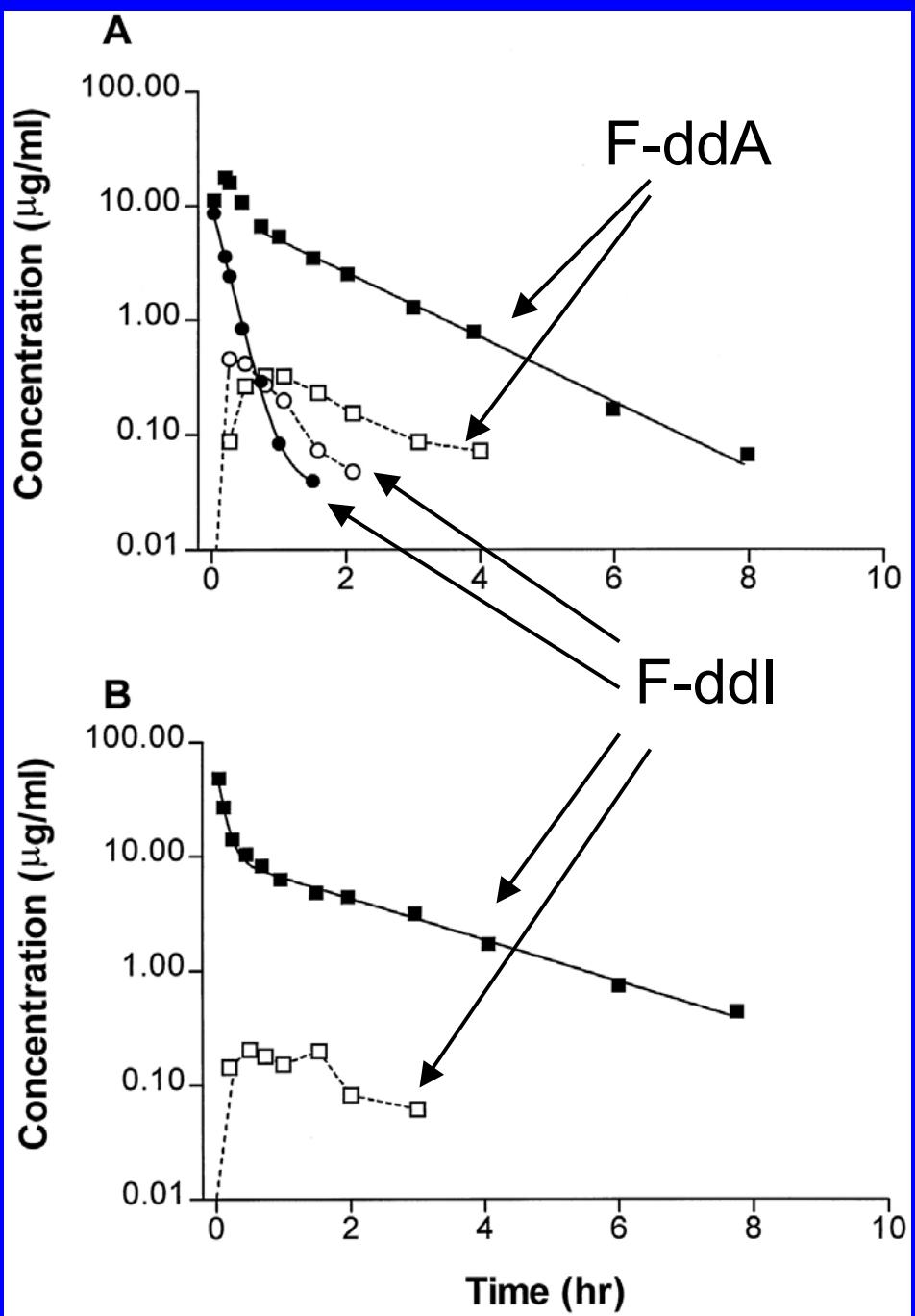
*Level 7 - 4.5 mg/kg*

*Level 8 - 6.4 mg/kg*

*(Arrows indicate days of current PK sampling)*



Plasma and  
CSF  
concentration  
versus time  
profiles in  
monkey after  
20 mg/kg i.v.  
push of FddA  
(panel A) or F-  
ddl (panel B)  
by  
HPLC/UV



JS Roth et al (1999)

Drug Metab Disp 27:1128-32

# LC/MS Sample Preparation

## Blood

1. Collect in Vacutainer containing
  - a) heparin
  - b)***2'-deoxycycoformycin*** (to make  $20\mu M$ )
2. Centrifuge @ 4000 Xg, 10 min

## Plasma

***0.5% Triton-X***

## Plasma (0.5 ml)

1. Add  $5\mu l$  1 mM **2-Cl-A** as internal standard
2. Vortex
3. Centrifuge in Amicon Centrifree system  
@ 2000 xg, 15-25 min

## Ultrafiltrate (~200 $\mu l$ )

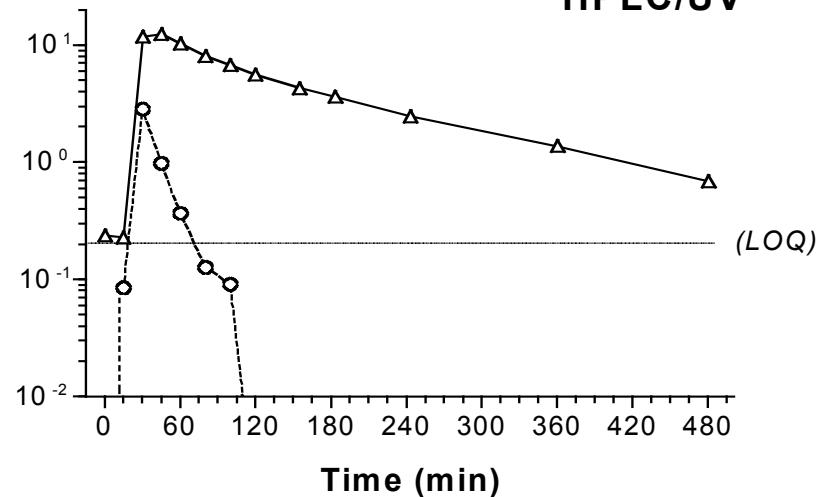
Inject  $10\mu l$  on narrow-bore HPLC

## HPLC - ESI - MS/MS Analysis

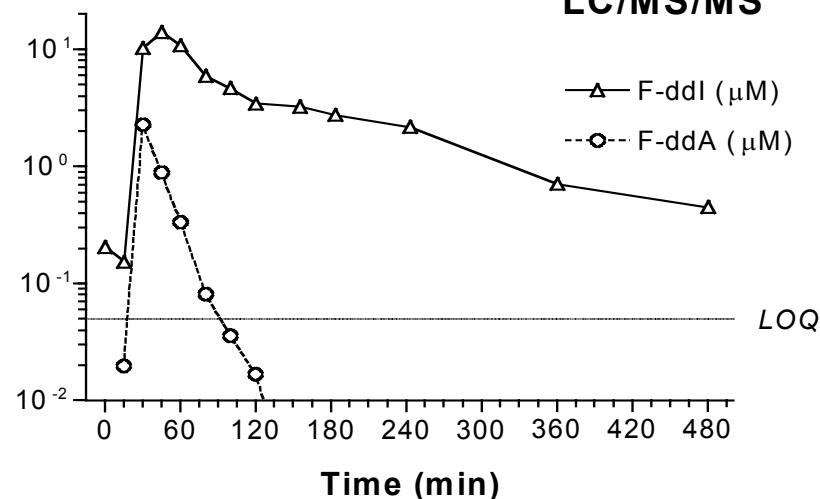
# Comparison of Methods for Plasma Analysis of F-ddA and F-ddI

3.2 mg/kg F-ddA  
Week 4 - capsules with food

## HPLC/UV



## LC/MS/MS



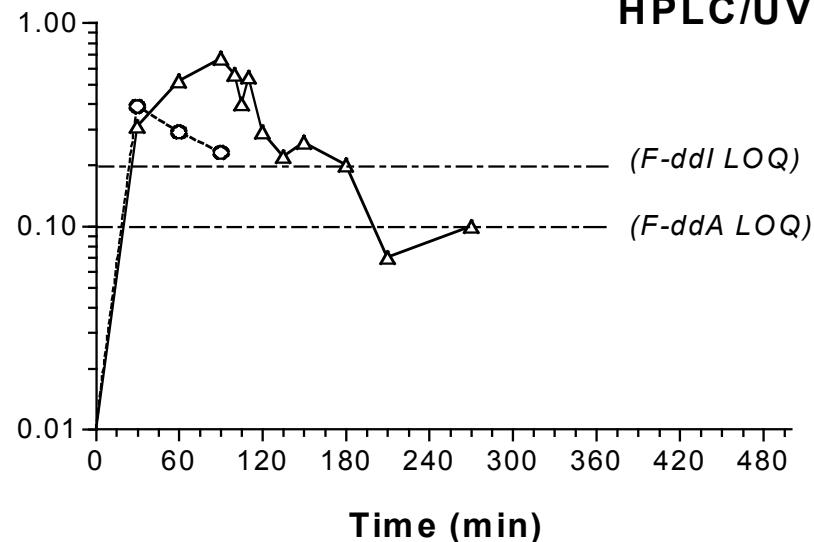
JS Roth et al (2000)

J. Mass Spectrom 35:1313-9

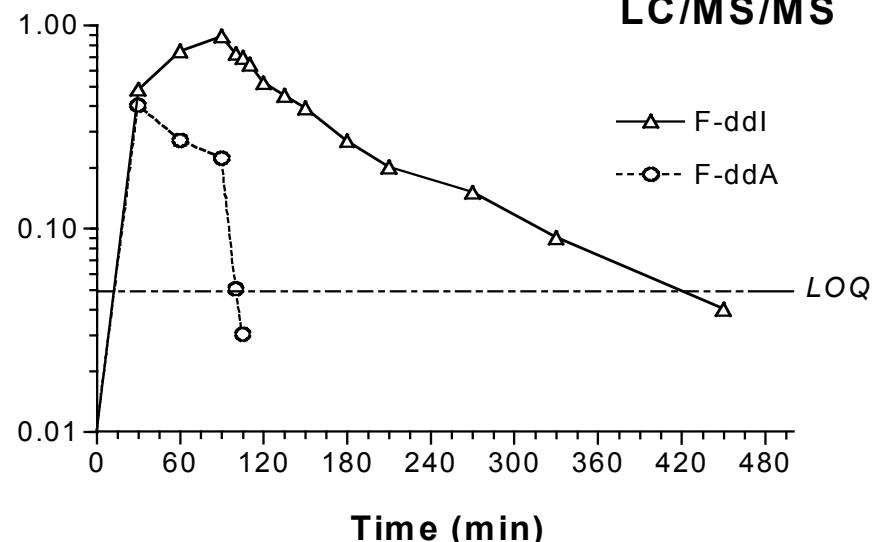
# Comparison of Methods for Plasma Analyses of F-ddA and F-ddI

0.2 mg/kg F-ddA

HPLC/UV



LC/MS/MS



JS Roth et al (2000)

J. Mass Spectrom 35:1313-9

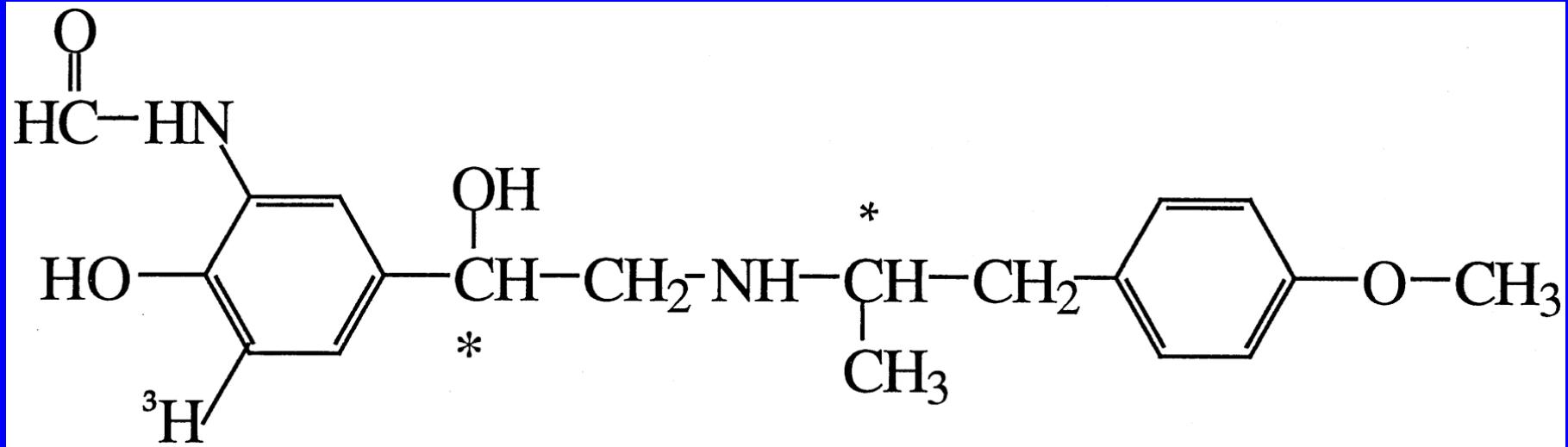
# Summary

- HPLC-UV
  - Measures both F-ddA and F-ddI
  - Primary assay for clinical samples
  - Adequate sensitivity for PK studies at therapeutic doses
- LC-ESI-MS-MS
  - Measures both F-ddA and F-ddI
  - Simple sample preparation and rapid analysis
  - High sensitivity and specificity
  - Backup assay for high volume of clinical samples

## Example 2 -Where Do Drugs Go?

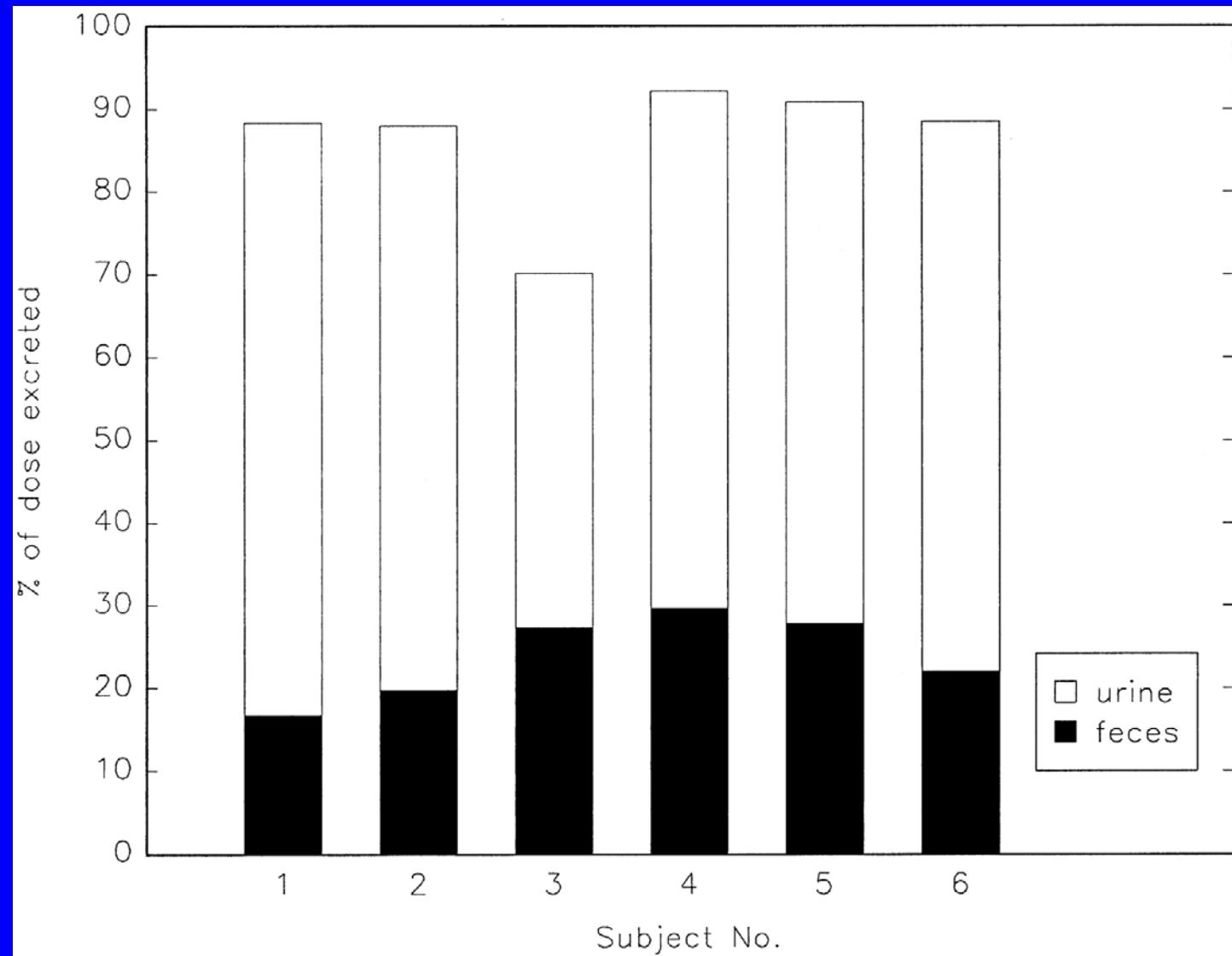
- Radiochemical tracers ( $^{14}\text{C}$ ,  $^3\text{H}$ )
  - requires availability of labeled drug
  - useful for mass balance determination- **Formoterol**
    - detection of protein adducts/localization (autoradiography)
- Non-radiochemical methods
  - Unique drug elements (fluorine, etc.) or structural property (fluorescence)
  - Specific atom or isotope detectors
    - Accelerator mass spectrometry (AMS) - detection of  $^{14}\text{C}$  at near natural background levels (K. Turteltaub et al.)
    - Combustion reaction isotope mass spectrometry (CRIMS) (F. Abramson et al.)

# Formoterol

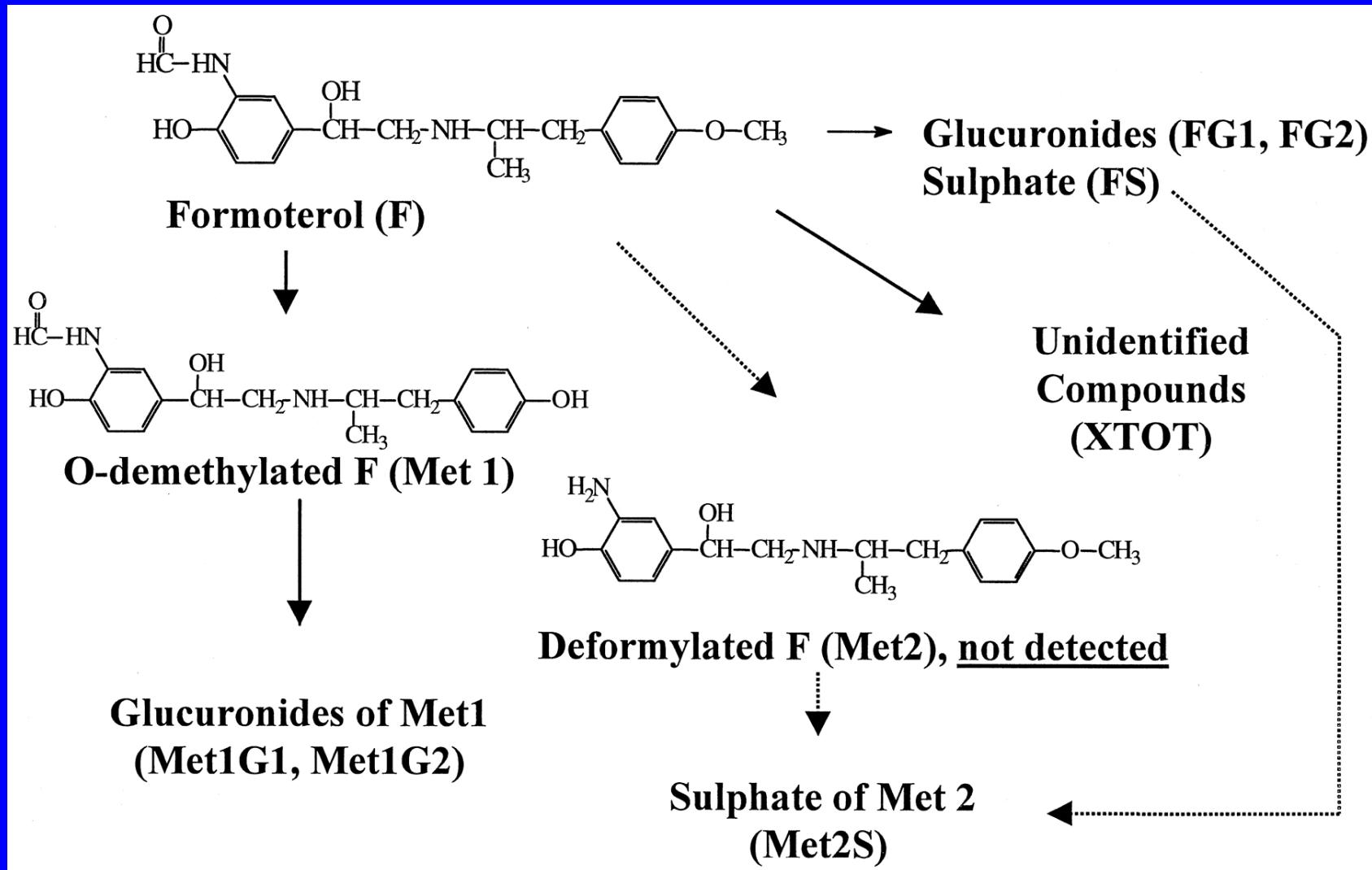


J. Rosenborg, P. Larsson, K. Tegnér, and G. Hallström  
Drug Metabolism & Disposition: 27, Issue 10, 1104-1116, 1999  
“Mass Balance and Metabolism of [<sup>3</sup>H]Formoterol in Healthy Men  
after Combined i.v. and Oral Administration-Mimicking Inhalation”

# Mass Balance: Recovery of Radioactivity

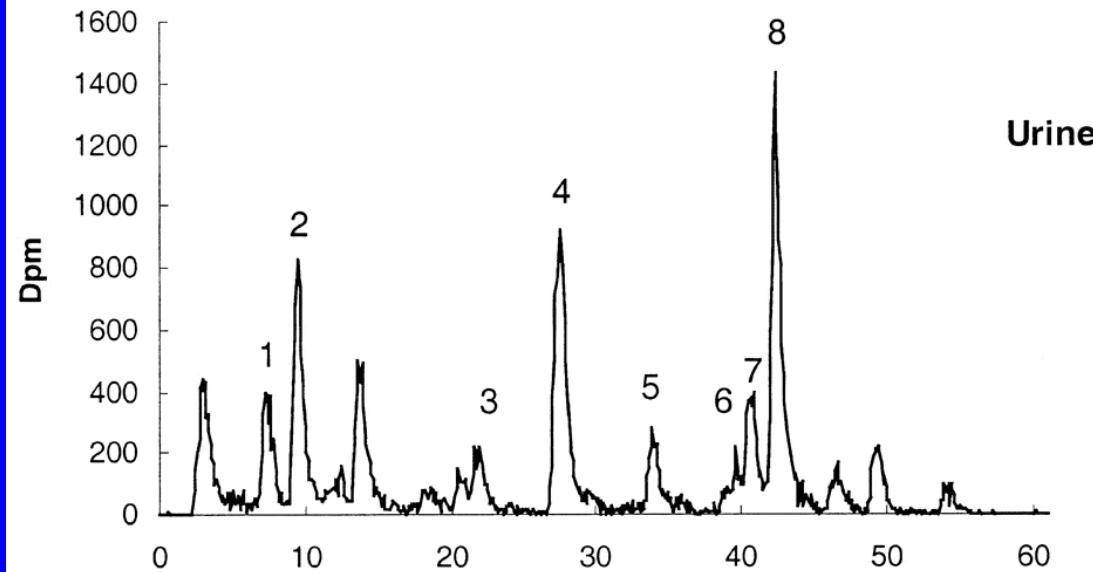
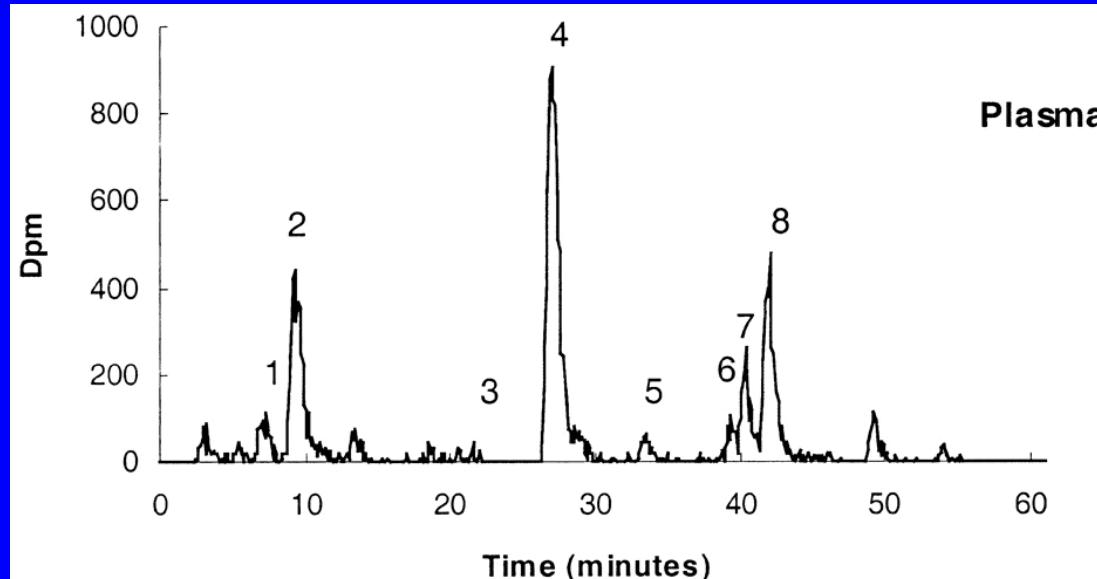


# Formoterol Metabolic Pathway



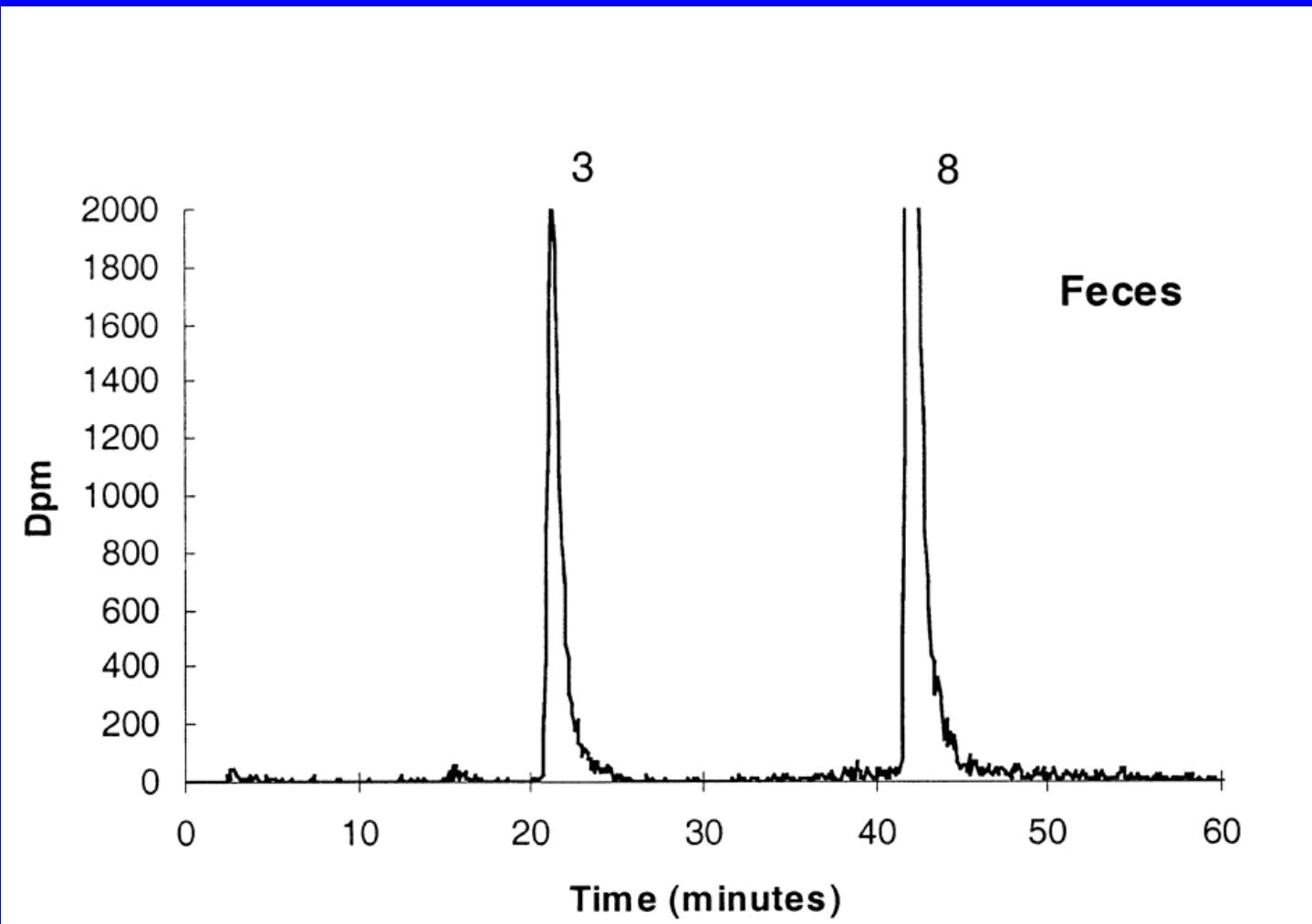
# Radiochromatography (1)

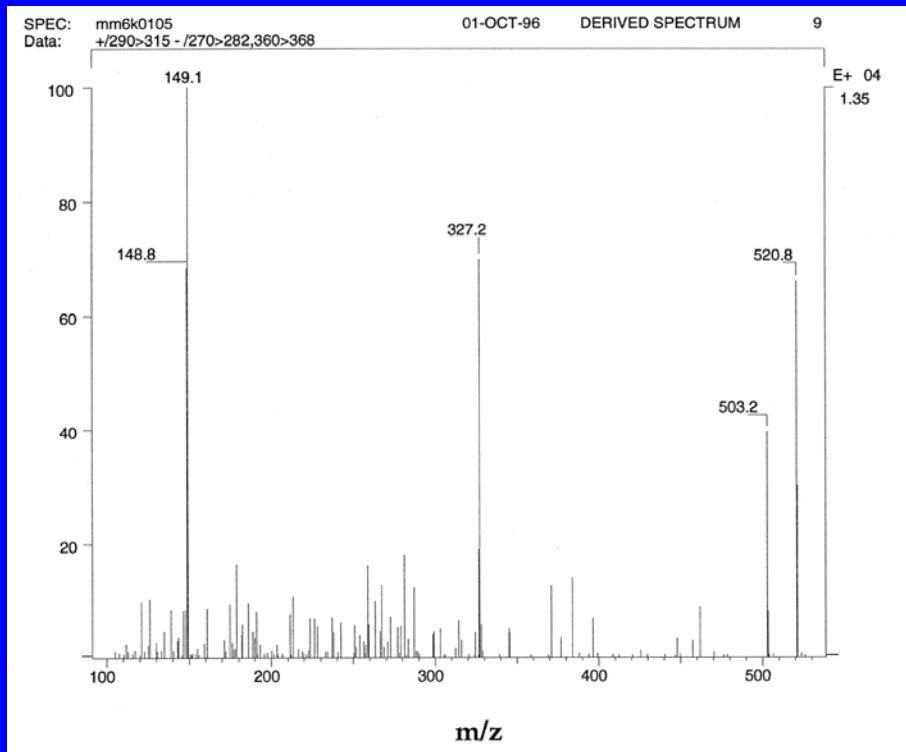
- 1, Met1G1
- 2, Met1G2
- 3, Met1
- 4, FG1
- 5, Met2S
- 6, FS
- 7, FG2
- 8, Formoterol.



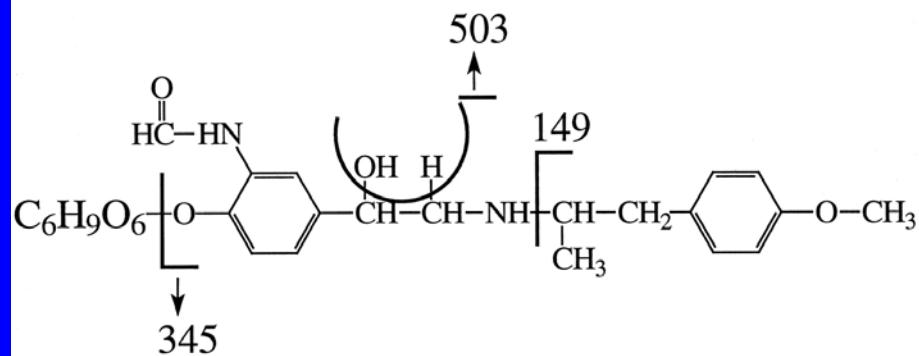
# Radiochromatography (2)

- 1, Met1G1
- 2, Met1G2
- 3, Met1
- 4, FG1
- 5, Met2S
- 6, FS
- 7, FG2
- 8, Formoterol.





# Formoterol Glucuronide (phenol, FG1) - LC/MS/MS



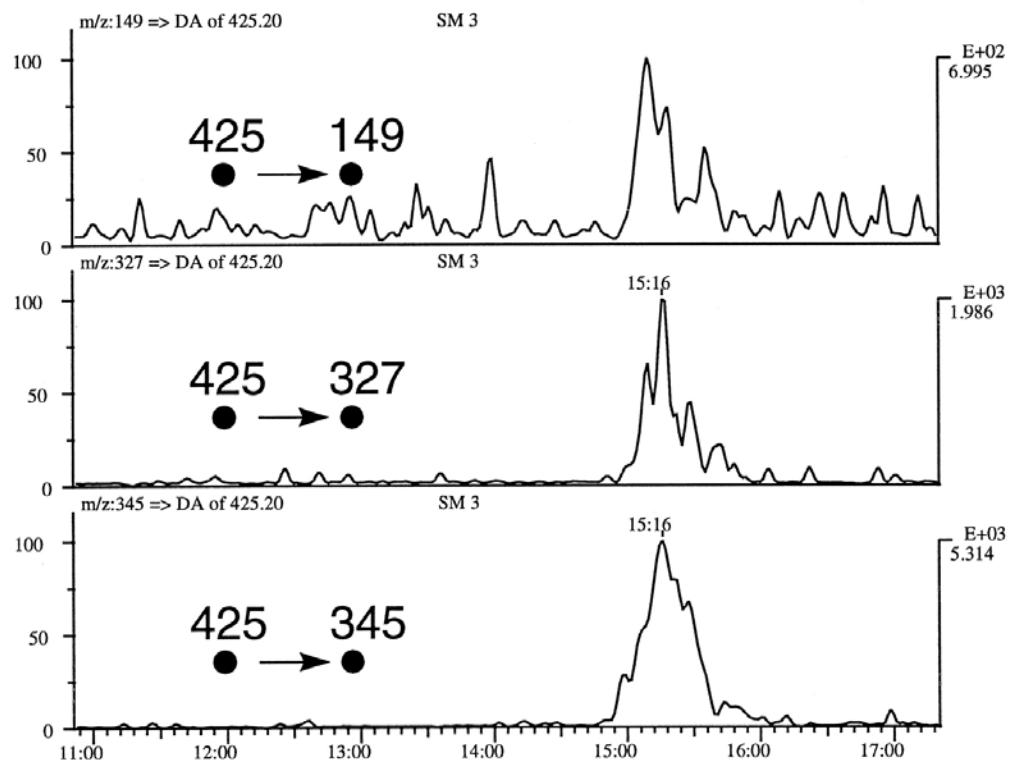
CHRO: mm6k1810

18-OCT-96

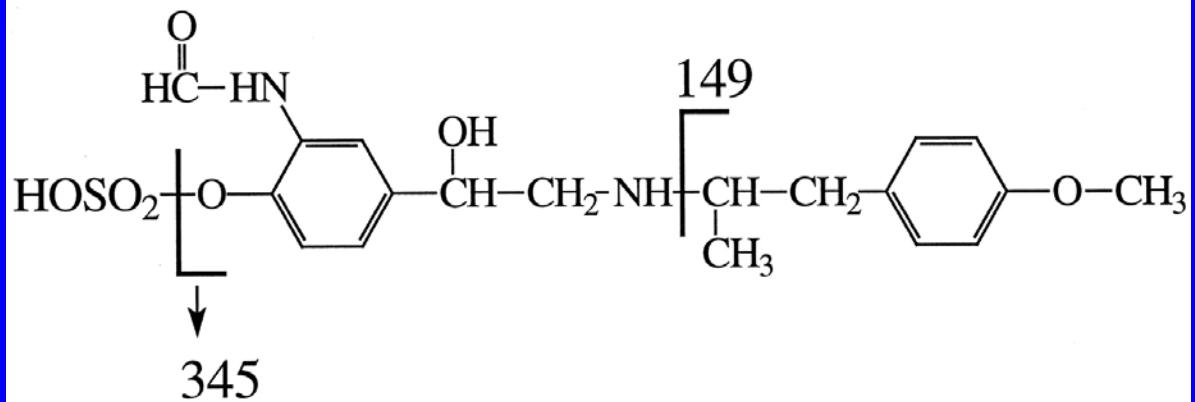
Elapse:

12:00.1

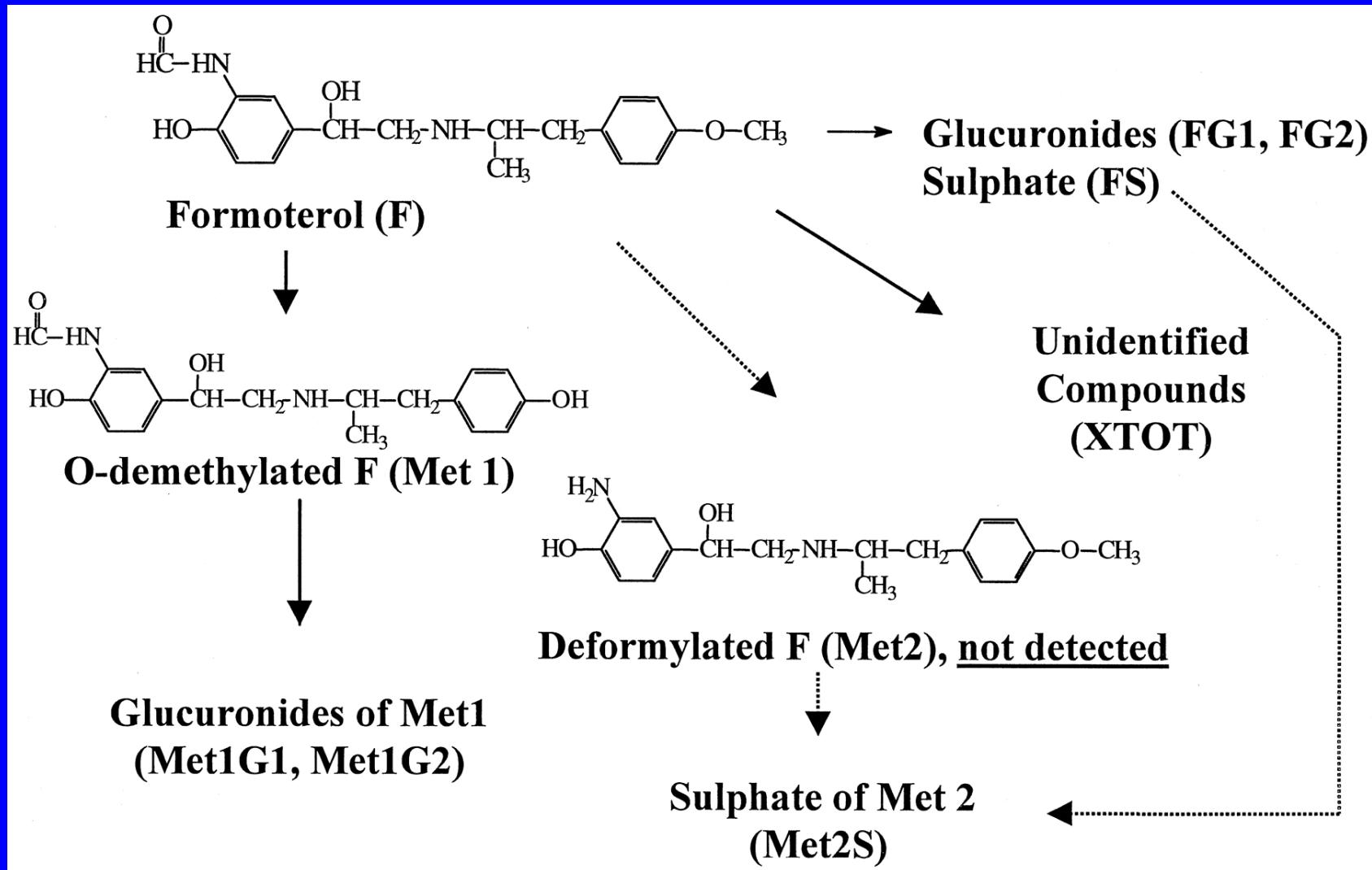
553



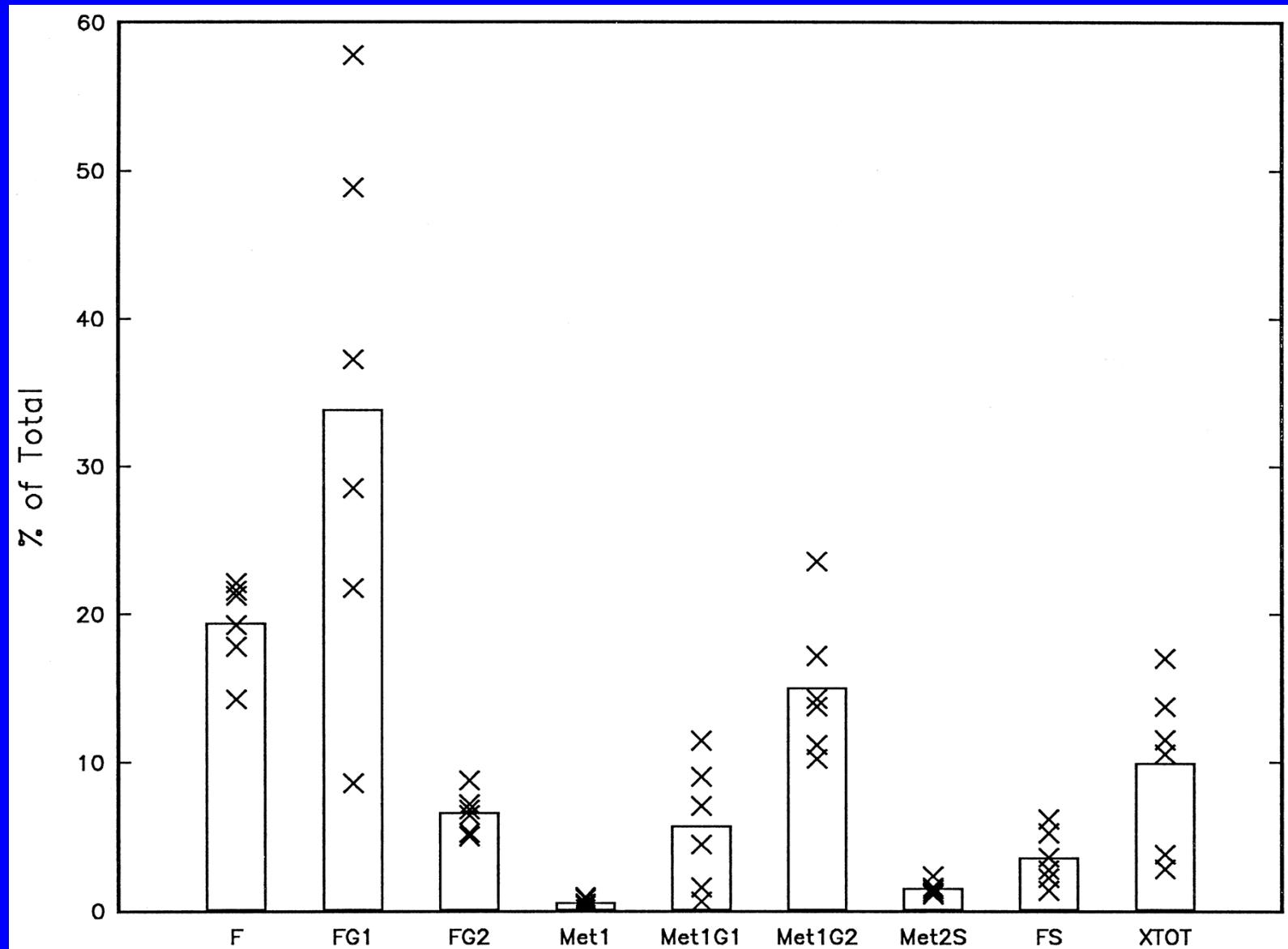
# Formoterol Sulfate - LC/MS/MS

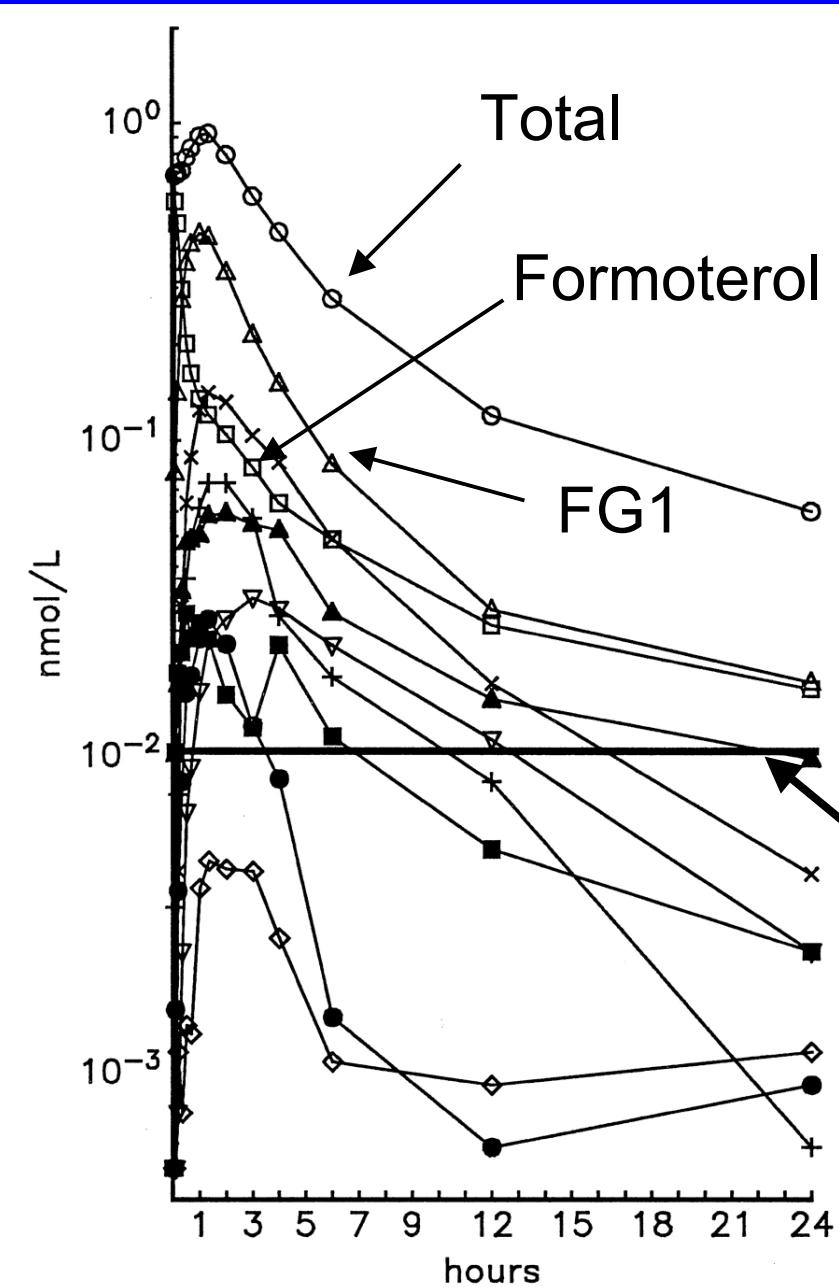


# Formoterol Metabolic Pathway



# Plasma AUC Formoterol Metabolites





Plasma -  
Conc Time  
Curves for  
Formoterol  
and  
Metabolites

LOQ

## References

- Tutorial for HPLC
  - <http://kerouac.pharm.uky.edu/asrg/hplc/HPLCMYTRY.HTML>
- Directory of Contract Research: *R&D Directions* (Engel Publishing, NJ)
- Prediction Software: [www.compudrug.com](http://www.compudrug.com) (pK, metabolites)
- Predictive value of in vitro model systems in toxicology: Davila, J.C., et al. Ann. Rev. Pharmacol. Toxicol. 1998;38: 63-96.
- Human Liver Slices: Olinga, P. et al., Drug Metab. Disposition 26:5-11, 1998
- Human Drug Metabolizing Enzymes:
  - Panvera ([www.panvera.com](http://www.panvera.com))
  - Human Biologics International ([www.humanbiologics.com](http://www.humanbiologics.com))
  - InVitro Tech ([www.invitrotech.com](http://www.invitrotech.com))
- HPLC-NMR: Lindon, J.C. et al., Drug Metab. Revs. 29:705-746 (1997)

## WWW Sites - Mass Spectrometry Information/Education

- <http://ull.chemistry.uakron.edu/classroom.html>
  - Excellent introductory tutorials in analytical methods including chromatography and mass spectrometry
- <http://base-peak.wiley.com>
  - Site with very useful links for mass spectrometry
- <http://homework.chem.uic.edu/MDL.HTM>
  - Organic chemistry tutorial, including spectroscopies
- <http://www.sisweb.com/mstools.htm>
  - Good calculation tools free ware (isotope calculations, elemental compositions)
- <http://www.asms.org/>
  - American Society for Mass Spectrometry for employment, educational activities, conferences